

JOURNAL OF
EXPERIMENTAL
BOTANY

AN OFFICIAL ORGAN OF THE
SOCIETY FOR
EXPERIMENTAL BIOLOGY

VOLUME I

OXFORD
AT THE CLARENDON PRESS
1950

Oxford University Press, Amen House, London E.C.4

GLASGOW NEW YORK TORONTO MELBOURNE WELLINGTON

BOMBAY CALCUTTA MADRAS CAPE TOWN

Geoffrey Cumberlege, Publisher to the University

PRINTED IN GREAT BRITAIN
AT THE UNIVERSITY PRESS, OXFORD
BY CHARLES BATEY, PRINTER TO THE UNIVERSITY

Editorial Committee

T. A. BENNET-CLARK (Editor)

E. ASHBY • G. E. BLACKMAN

R. BROWN • F. G. GREGORY

W. O. JAMES • W. H. PEARSALL

R. D. PRESTON • M. THOMAS

CONTENTS

NUMBER 1

F. G. GREGORY, F. L. MILTHORPE, H. L. PEARSE, and the late H. J. SPENCER: Experimental Studies of the Factors Controlling Transpiration. I. Apparatus and Experimental Technique	I
F. G. GREGORY, F. L. MILTHORPE, H. L. PEARSE, and the late H. J. SPENCER: Experimental Studies of the Factors Controlling Transpiration. II. The Relation Between Transpiration Rate and Leaf Water Content	15
O. V. S. HEATH: Studies in Stomatal Behaviour. V. The Role of Carbon Dioxide in the Light Response of Stomata.	29
A. A. BENSON and M. CALVIN: The Path of Carbon in Photosynthesis. VII. Respiration and Photosynthesis	63
I. MANTON: Demonstration of Compound Cilia in a Fern Spermatozoid by Means of the Ultra-violet Microscope	69
A. ALLSOPP: Further Studies on the Acid Metabolism of <i>Aspergillus niger</i> . The Formation and Utilization of Oxalic Acid	71
R. BROWN and J. F. SUTCLIFFE: The Effects of Sugar and Potassium on Extension Growth in the Root	88
W. T. WILLIAMS: Studies in Stomatal Behaviour. IV. The Water-relations of the Epidermis	114

NUMBER 2

R. SCOTT RUSSELL and R. P. MARTIN: Studies with Radioactive Tracers in Plant Nutrition. I. Problems in the use of Radioactive Tracers	133
R. P. MARTIN and R. SCOTT RUSSELL: Studies with Radioactive Tracers in Plant Nutrition. II. The Estimation of Radioactive Tracers	141
F. G. GREGORY and B. SAMANTARAI: Factors concerned in the Rooting Responses of Isolated Leaves	159
D. J. D. HOCKENHULL: Studies in the Metabolism of Mould Fungi. I. A Preliminary Study of the Metabolism of Carbon, Nitrogen, and Sulphur by <i>Aspergillus nidulans</i>	194
JOYCE A. BENTLEY: An Examination of a Method of Auxin Assay using the Growth of Isolated Sections of <i>Avena</i> Coleoptiles in Test Solutions	201

Contents

R. D. PRESTON and K. SINGH: The Fine Structure of Bamboo Fibres. I. Optical Properties and X-Ray Data	214
O. V. S. HEATH and F. L. MILTHORPE: Studies in Stomatal Behaviour. V. The Role of Carbon Dioxide in the Light Response of Stomata.	227
P. E. WEATHERLEY: A Convenient Volumenometer for Biological Work	244

NUMBER 3

R. BROWN and D. BROADBENT: The Development of Cells in the Growing Zones of the Root	249
H. W. B. BARLOW: Studies in Abscission. I. Factors affecting the Inhibition of Abscission by Synthetic Growth-Substances	264
E. C. HUMPHRIES: The Absorption of Ions by Excised Root Systems. I. Apparatus and Preliminary Experiments	282
F. J. TAYLOR: Oxidative Assimilation of Glucose by <i>Scenedesmus quadricauda</i>	301
M. A. ABOU RAYA: The Rate of Water-loss from Stripped Leaves	322
W. W. SCHWABE: Factors controlling Flowering of the Chrysanthemum. I. The Effects of Photoperiod and Temporary Chilling	329
R. D. PRESTON, P. H. HERMANS, and A. WEIDINGER: The Crystalline- Non-Crystalline Ratio in Celluloses of Biological Interest	344
M. HOLDSWORTH and O. V. S. HEATH: Studies in the Physiology of the Onion Plant. IV. The Influence of Day-Length and Temperature on the Flowering of the Onion Plant	353

INDEX TO VOLUME I

ALLSOPP, A. Further Studies on the Acid Metabolism of <i>Aspergillus niger</i> . The Formation and Utilization of Oxalic Acid	71
BARLOW, H. W. B. Studies in Abscission. I. Factors affecting the Inhibition of Abscission by Synthetic Growth-Substances	264
BENSON, A. A. and CALVIN, M. The Path of Carbon in Photosynthesis. VII. Respiration and Photosynthesis	63
BENTLEY, JOYCE A. An Examination of a Method of Auxin Assay using the Growth of Isolated Sections of <i>Avena</i> Coleoptiles in Test Solutions.	201
BROADBENT, D., see BROWN, R.	
BROWN, R. and SUTCLIFFE, J. F. The Effects of Sugar and Potassium on Extension Growth in the Root	88
BROWN, R. and BROADBENT, D. The Development of Cells in the Growing Zones of the Root.	249
CALVIN, M., see BENSON, A. A.	
GREGORY, F. G., MILTHORPE, F. L., PEARSE, H. L., and the late SPENCER, H. J. Experimental Studies of the Factors Controlling Transpiration. I. Apparatus and Experimental Technique	I
GREGORY, F. G., MILTHORPE, F. L., PEARSE, H. L., and the late SPENCER, H. J. Experimental Studies of the Factors Controlling Transpiration. II. The Relation Between Transpiration Rate and Leaf Water Content	15
GREGORY, F. G. and SAMANTARAI, B. Factors concerned in the Rooting Responses of Isolated Leaves	159
HEATH, O. V. S. Studies in Stomatal Behaviour. V. The Role of Carbon Dioxide in the Light Response of Stomata.	29
HEATH, O. V. S. and MILTHORPE, F. L. Studies in Stomatal Behaviour. V. The Role of Carbon Dioxide in the Light Response of Stomata.	227
HEATH, O. V. S., see HOLDSWORTH, M.	
HERMANS, P. H., see PRESTON, R. D.	
HOCKENHULL, D. J. D. Studies in the Metabolism of Mould Fungi. I. A Preliminary Study of the Metabolism of Carbon, Nitrogen, and Sulphur by <i>Aspergillus nidulans</i>	194
HOLDSWORTH, M. and HEATH, O. V. S. Studies in the Physiology of the Onion Plant. IV. The Influence of Day-Length and Temperature on the Flowering of the Onion Plant	353
HUMPHRIES, E. C. The Absorption of Ions by Excised Root Systems. I. Apparatus and Preliminary Experiments	282
MANTON, I. Demonstration of Compound Cilia in a Fern Spermatozoid by Means of the Ultra-violet Microscope	69
MARTIN, R. P. and RUSSELL, R. SCOTT. Studies with Radioactive Tracers in Plant Nutrition. II. The Estimation of Radioactive Tracers	141
MARTIN, R. P., see RUSSELL, R. SCOTT	
MILTHORPE, F. L., see GREGORY, F. G.	
MILTHORPE, F. L., see HEATH, O. V. S.	
PEARSE, H. L., see GREGORY, F. G.	
PRESTON, R. D. and SINGH, K. The Fine Structure of Bamboo Fibres. I. Optical Properties and X-Ray Data	214

Index to Volume I

PRESTON, R. D., HERMANS, P. H., and WEIDINGER, A. The Crystalline-Non-Crystalline Ratio in Celluloses of Biological Interest	344
RAYA, M. A. ABOU. The Rate of Water-loss from Stripped Leaves	322
RUSSELL, R. SCOTT and MARTIN, R. P. Studies with Radioactive Tracers in Plant Nutrition. I. Problems in the use of Radioactive Tracers	133
RUSSELL, R. SCOTT, see MARTIN, R. P.	
SAMANTARAI, B., see GREGORY, F. G.	
SCHWABE, W. W. Factors controlling Flowering of the Chrysanthemum. I. The Effects of Photoperiod and Temporary Chilling	329
SINGH, K., see PRESTON, R. D.	
SPENCER, H. J., the late, see GREGORY, F. G.	
SUTCLIFFE, J. F., see BROWN, R.	
TAYLOR, F. J. Oxidative Assimilation of Glucose by <i>Scenedesmus quadricauda</i>	301
WEATHERLEY, P. E. A Convenient Volumenometer for Biological Work	244
WEIDINGER, A., see PRESTON, R. D.	
WILLIAMS, W. T. Studies in Stomatal Behaviour. IV. The Water-relations of the Epidermis	114

Experimental Studies of the Factors Controlling Transpiration

I. APPARATUS AND EXPERIMENTAL TECHNIQUE¹

F. G. GREGORY, F. L. MILTHORPE, H. L. PEARSE, AND
THE LATE H. J. SPENCER

*From the Research Institute of Plant Physiology, Imperial College of Science and
Technology, London*

INTRODUCTION

PREVIOUS work on the factors concerned in controlling transpiration has suffered from excessive concentration on the physical aspects of the process. Ever since the classical experiments of Brown and Escombe (1900) called attention to the nature of diffusive processes in the leaf, experimental work has mainly been confined to investigations of evaporation through pores in diaphragms simulating the epidermis of the leaf as closely as possible. Biological aspects have not been investigated with the same persistency. This may, in part at least, be due to the intrinsic difficulties of exploring the effects of the various factors concerned. Before these may be investigated with any prospect of success, it is necessary to devise methods for changing at will the water content of the leaf and estimating the change in stomatal aperture without destroying the leaf or interfering with its normal functioning.

In this Institute, work along these lines was begun long ago by Knight, who first exploited the possibilities of studying with a porometer the relation between transpiration and stomatal aperture. His classical work on *Eupatorium adenophorum* has now found its way into most text-books of plant physiology, but a continuation of the work, so brilliantly initiated by Knight, awaited a period of 12 years, at which time work on transpiration was recommenced in this Institute. The necessary methods have been devised for carrying out experimental studies and a large body of data has been assembled. These data will be presented in this series of papers.

The various aspects of research in transpiration may be divided into five main categories:

- i. Investigations of physical factors of diffusion as determined by the structure of the leaf and environmental factors, such as temperature, light, humidity, and air flow.
- ii. The regulatory role of the stomata and the study of stomatal behaviour.
- iii. The part played by changing leaf water content in determining transpiration rate.

¹ This paper includes work embodied in theses submitted to the University of London for degrees of Ph.D. by H. L. P. (1936), H. J. S. (1938), and F. L. M. (1948).

- iv. Permeability changes associated with wilting and the problems of drought resistance.
- v. Ecological aspects of water loss by plants, such as the transpiration ratio.

No attempt will be made in this paper to discuss the vast literature dealing with these different aspects of transpiration. At various times reviews have appeared, of which the following may be cited: Knight (1917*a*), Burgerstein (1920), Scarth (1927), Maximov (1929), Seybold (1929). Citations from the literature relevant to the subject under discussion will be included in the separate papers of this series. In this paper discussion will be confined to technique.

Earlier experimental studies in this field have been limited to the effects of the single factors, and their interactions have almost entirely been neglected. Even in the classical experiments of Knight (1917*b*, 1922), in which water uptake and loss and stomatal aperture were studied on leafy shoots, the effect of water content was followed only during the falling phase on stoppage of the water-supply and also over relatively small changes resulting from temporarily stopping the air stream. Unfortunately the actual water changes in the shoot were not accurately determined, and the use of a shoot as an experimental unit did not provide a sensitive measure of the water content of the transpiring leaves. Furthermore, the stomatal aperture was measured on a portion of a leaf permanently enclosed in a porometer cup, and it was assumed that the behaviour of these stomata gave a true picture of the behaviour on the rest of the leaf. Stålfelt's (1929, 1932) experiments using detached portions of the lamina hung directly on a balance did not suffer from this disadvantage, since efforts were made to determine the stomatal aperture by direct observations of portions of the lamina removed from control leaves kept under the same conditions. From the very nature of this method, however, the number of stomata examined was always small, and no account was taken of the possible shock reaction of the stomata following excision of the sampled portion used for measurement.

In the experiments to be described in this series of papers the aim has been to keep the water-supply as well as the other external factors under control. Single leaves have throughout been used, and these have been taken through a cycle of water-content changes. The leaf, kept under constant conditions of light, temperature, and air flow, was allowed to wilt to a predetermined degree by suitably interrupting the water-supply and was then allowed to recover the turgid condition by rapidly re-establishing the water-supply. Meanwhile, the transpiration rate was determined by absorbing and weighing the water vapour lost, and the changes in stomatal aperture were followed with a resistance porometer (Gregory and Pearse, 1934). Water absorption was measured as long as water-supply was maintained. The difference in weight of water absorbed and lost could thus be determined throughout the cycle of change, and by estimating the absolute water content of the leaf at the end the water content was known at any previous stage of the experiment. In

some experiments leaves were submitted to induced cycles of stomatal opening and closure by exposing to light and darkness, the concurrent changes in water uptake and loss also being determined. In this way the effects of the factors of stomatal aperture and water content on the transpiration of the leaf were followed.

These experiments have been confined to two types of leaves, viz. *Pelargonium zonale* var. 'Paul Crampel' and wheat (*Triticum vulgare* vars. 'Bencubbin' and 'Charter').

DETAILS OF APPARATUS

1. Leaf chambers

(a) In the first experiments carried out by Pearse on *Pelargonium* a very simple leaf chamber was used (Fig. 1). This consisted of two thick Petri dishes of equal size, one of which was provided with three $\frac{1}{2}$ -in. holes fitted with

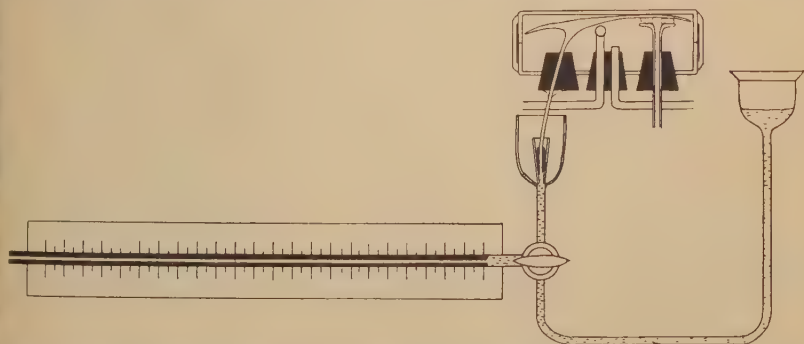


FIG. 1. Diagram of longitudinal section of leaf chamber as first used by Pearse for *Pelargonium*, showing arrangement of leaf, porometer cup, and water-supply. For explanation, see text.

rubber stoppers. Through one stopper passed inlet and outlet holes to carry the air supply; the second stopper consisted of two halves enclosing an inclined aperture through which the leaf petiole passed; and the third stopper held the porometer cup. The second Petri dish rested accurately on the top of the first, and the two dishes were then sealed to give an airtight joint with a strip of inch-wide medical adhesive tape. This was then coated with a layer of paraffin wax. A simple porometer cup was so adjusted that the leaf in contact with the lid was pressed down on to a washer of 35 per cent. gelatine in the porometer cup just sufficiently to secure an airtight joint. The joint between the split stopper and the petiole of the leaf was made secure with luting wax consisting of equal parts of beeswax, paraffin wax, and vaseline. The split stopper enabled the leaf to be enclosed in the chamber while still attached to the plant, if so desired.

(b) For the experiments on *Pelargonium* carried out by Spencer the leaf chamber was redesigned to enable a detachable porometer cup to be used. The chamber (Fig. 2) consisted of a shallow cylindrical brass box with a

circular lid of plate glass cemented into a solid brass ring *a*, which screwed down on the top of the chamber, the joint being made airtight with a greased rubber washer. By means of paraffin wax *b* the internal dimensions were reduced to 12 cm. diameter by 1.5 cm. deep. The base was supplied with three 1-in. holes fitted with rubber stoppers. Through the central one passed the inlet tube *c*, while through holes in the two other stoppers (which do not lie in the section of Fig. 2) passed respectively a thermometer and the leaf petiole. The bulb

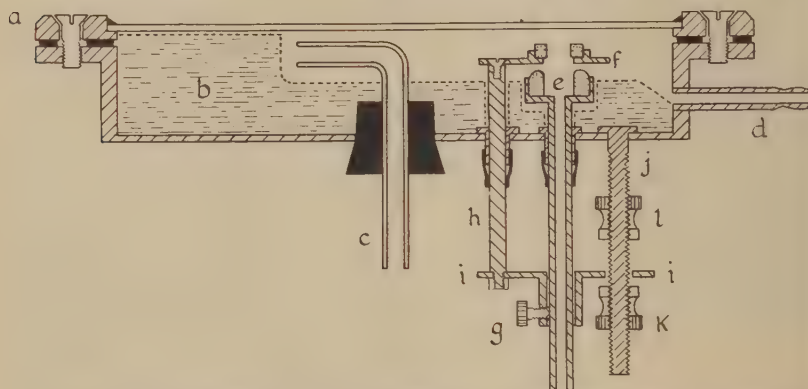


FIG. 2. Section of leaf chamber as used by Spencer for *Pelargonium* leaves. *a*, brass ring holding the glass lid; *b*, paraffin wax; *c*, inlet tube; *d*, outlet tube for air stream; *e*, lower porometer cup; *f*, upper porometer cup; *g*, thumbscrew locking the lower porometer cup; *h*, one of the brass rods supporting the upper porometer cup from the circular brass plate *i*; this plate moves vertically on the bolt *j* and is locked in position by the nuts *k* and *l*. For further explanation, see text.

of the thermometer projected just above the wax floor and was protected from direct radiation by a cap of copper foil. The hole through the leaf stopper was inclined, so that the lamina lay horizontal when the petiole was in position. The brass outlet tube *d* was soldered into the side of the chamber diametrically opposite to the inlet, which was bent as shown in the figure. A test with smoke indicated that this arrangement caused the air stream, after impinging upon the circular wax side, to spread out and flow evenly over the whole of the leaf before reaching the outlet. The volume of the chamber was 170 c.c.

(c) In the experiments carried out by Milthorpe with wheat leaves the leaf chamber as first used was essentially similar to that used by Spencer, except that the dimensions of the wheat leaf demanded a rectangular shape. The chamber consisted of a brass box, of dimensions $26 \times 1.75 \times 1.5$ cm. provided with a glass lid; it was approximately half-filled with paraffin wax to reduce the volume to the smallest convenient size. To secure an airtight joint the glass lid rested on a broad flange coated with a luting wax consisting of vaseline and beeswax. The effective volume of the chamber was 30.4 c.c.

The design of the leaf chamber was later modified; the chamber as now used is shown in Fig. 3. The glass lid was replaced by a sheet of perspex *a*,

which was pressed by small G-clamps against the flange of the box, being made airtight with a smear of vaseline. To secure a more adequate stream-line flow, the inlet *c* and outlet *d* tubes were splayed out into a funnel shape so that the air spread evenly across the chamber. In all the earlier experiments the leaf within the chamber was pressed between the washer of the porometer cup and the lid. The main air flow was therefore over the lower surface of the leaf and flow over the upper surface was impeded in the region of the porometer

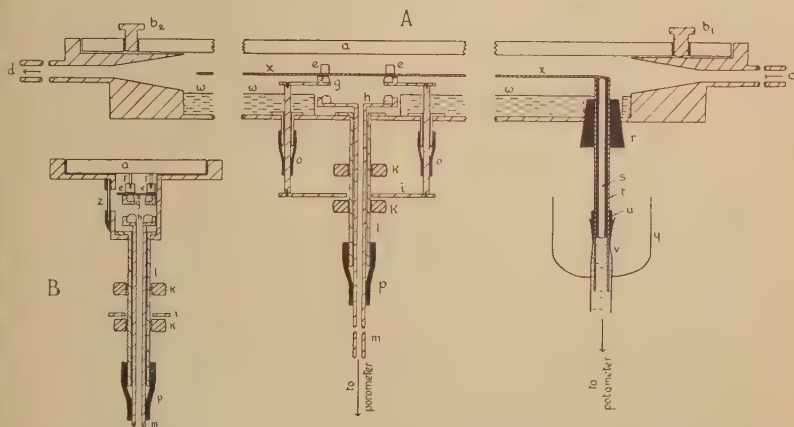


FIG. 3. Sections of leaf chamber used by Milthorpe for wheat leaves. A, median longitudinal section of portion of chamber with leaf fitted showing general construction of chamber and arrangement for water-supply, and median longitudinal section of one of the porometer cups; B, cross-section of chamber through centre of one of the porometer cups. *a*, perspex lid; *b*, lifting-screws for removal of lid from chamber; *c* inlet and *d* outlet tubes for air stream; *e*, perspex frame holding leaf on porometer cup and attached to the lid *a* by the perspex pillars *f*; *g* and *h*, upper and lower porometer cups supporting gelatine washers; *i*, rectangular brass plate supporting upper porometer cup; *k*, milled nuts for moving and locking plate *i*; *l*, threaded tube on which plate *i* moves freely; *m*, outlet tube from porometer cup; *n*, valve-rubber tubing; *o*, pressure tubing; *p*, split rubber stopper; *r*, glass rod over which valve tubing is stretched supporting leaf sheath *t*; *u*, outer sleeve of valve tubing; *v*, tapered glass tube connected to potometer; *w*, paraffin wax; *x*, leaf; *y*, outer jacket of potometer; *z*, glass window sealed to side of chamber.

cup. This was obviously undesirable, so arrangements were made to secure the leaf centrally in the chamber and thus ensure a symmetrical flow of air over both surfaces. The details of this modification are discussed in the section on porometer-cup design. This latest chamber contained two porometer cups placed at one-third and two-thirds of the length of the chamber. The dimensions of the chamber were $38.0 \times 1.3 \times 1.35$ cm., giving an effective volume of 63.6 c.c. A rim of brass surrounded the flange on which the lid rested and held the latter exactly in position. Two screws (*b*), one at each end, assisted the removal of the lid from the chamber. The chamber was partly filled with paraffin wax *w* to the level of the top of the lower porometer cups, which could be drawn down into recesses in the wax cut for this purpose. The leaf *in situ*

was supported centrally in the chamber by the stopper through which the leaf sheath was inserted, by the two porometer cups, and also at the distal end of the chamber by a small horizontally placed brass rod.

2. *Design of porometer cups*

(a) In the first experiments of Pearse the porometer cup was of the traditional design, consisting of a flanged glass tube (Fig. 1). Contact with the leaf was made by means of a washer of 35 per cent. gelatine cut from a sheet by cork-borers of standard size, so that the same size washer was always employed. The porometer cup was attached to a three-way tap, the other limbs of which led to the manometer and through a fixed capillary resistance to a constant-pressure aspirator. While a porometer reading was being taken, the air stream through the chamber was temporarily stopped, so that the pressure in the leaf chamber fell to atmospheric.

(b) In Spencer's experiments a detachable porometer cup was used for the first time. Its construction is shown diagrammatically in Fig. 2. The porometer cup *e* consisted of a flanged brass tube of 0.9 cm. diameter carrying a gelatine washer which could be brought into an airtight connexion with the triangular plate *f*. This plate had a central hole around which a second gelatine washer rested, within a ring on the upper surface. This washer made contact with the under surface of the leaf. Both washers were of 35 per cent. gelatine dissolved in a mixture of 2 parts glycerine to 1 part water by weight. This mixture was first used by Newton (1936) to prevent drying of the washer. While a porometer reading was in progress the cup was kept in position against the triangular plate by means of the thumb-screw *g*. After completing the reading the cup was drawn down again into the recess cut into the paraffin wax to receive it.

The triangular plate was attached by screws at its corners to three steel rods, of which only one, *h*, is visible in Fig. 2. These bolted into a circular brass plate *i* bearing the thumb-screw *g*. The steel rods and the stem of the porometer cup passed through flanged holes in the base of the chamber. Sleeves of greased rubber tubing stretched over the flanges rendered these four sliding joints airtight. A bolt *j*, which was screwed and soldered into the chamber base, passed freely through a hole in the circular plate, so that, by means of two milled nuts *k* and *l*, the pair of connected plates could be screwed up or down, lateral movement being prevented by the accurate fit of the sliding joints.

The porometer cup was connected to a three-way tap, one arm of which was free to the air and the other connected by means of a T-piece to a water manometer and, through a fixed capillary resistance, to a constant-pressure aspirator. The capillary was protected from water vapour by calcium chloride tubes at both its ends, and the entry of dust was prevented by a light wad of cotton-wool. The fixed capillary resistance used throughout was 3.2 Gregory and Pearse units.¹ The area of the leaf over the porometer washer was determined at the end of each experiment by measuring the mean internal

¹ One Gregory and Pearse unit = $3.77 \times 10^8 \text{ cm.}^{-3}$ (Heath, 1939).

diameter of the ring of grease left on the lamina after its removal from the chamber; it was always approximately 40 sq. mm. in the experiments with this leaf chamber. The constant-pressure aspirator gave a head of approximately 11 cm. water, the exact value being measured at the beginning and conclusion of each experiment.

(c) The detachable porometer cup as first used by Milthorpe was essentially similar to that employed by Spencer. It was, however, rectangular in shape, as is commensurate with the dimensions of the wheat leaf. Both the upper and lower cups were made of brass and were $2.3 \times 1.0 \times 0.2$ cm. in size. Both cups carried gelatine washers; the internal dimensions of the washer in contact with the leaf were 1.5×0.25 cm.

Reference has already been made to later modifications in design of the porometer cup which avoided contact of the leaf with the lid of the chamber (Section 1c). The two porometer cups were of similar construction to those used previously, but the leaf was supported against a perspex frame instead of directly against the lid of the chamber (Fig. 3). The perspex frame had external dimensions of 2.4×0.9 cm. and internal dimensions of 1.8×0.3 cm. It was 0.3 cm. thick and was attached to the lid of the chamber by means of six perspex pillars 0.1 cm. in diameter. The pillars were screwed into the frame and were firmly fixed to the perspex lid of the chamber by means of perspex cement. The chamber lid always fitted exactly in the same position, this being assured by a brass flange surrounding the edge of the casing on which the lid rested. Each perspex frame was so placed that, when the lid was in position, the leaf was held firmly over the gelatine washer, the rectangular hole in the frame being exactly above the corresponding hole in the gelatine washer.

The porometer cups were placed towards one side of the chamber so that one of the long sides of the cup rested against the side of the chamber, thus preventing the lower cup from rotating during manipulation. The upper cup was screwed to two brass rods which passed through flanged holes in the base of the chamber and were screwed to a rectangular plate *i*. The rectangular plate had a central hole which fitted loosely over a central threaded tube *l* protruding from the base of the chamber. Milled nuts placed above and below the rectangular plate enabled the plate and the upper porometer cup fixed to it to be moved up and down and to be locked firmly in position when so required. The outlet tube *m* from the lower porometer cup *h* passed through the centre of the threaded tube *l*. Valve-rubber tubing *o* placed over the brass rods and their respective flanged tubes made the joints airtight. The junction of the outlet tube *m* from the lower porometer cup and the central brass tube *l* in which it was guided was made airtight by means of a length of pressure tubing lubricated with silicone grease. The two porometer cups were connected through a three-way tap to the resistance porometer (Fig. 5).

The resistance porometer used in this work incorporated the improvements introduced by Heath (1939, 1941). Two capillary resistances were used, one 0.299 and the other 1.17 Gregory and Pearse units in magnitude. In later work

the constant-pressure aspirator was replaced by a gasometer which provided a steady pull and obviated the fluctuations of pressure in the manometers as the bubble of air was released in the aspirator. The two manometers of the porometer were read at each determination by means of horizontal reading microscopes. Liquid paraffin of density 0.83 or dekaline was used as a manometer fluid; the use of these hydrophobic liquids dispenses with the necessity of constantly cleaning the manometers as is the case if water is used.

3. *The control of water-supply*

The method used for providing the detached leaf of *Pelargonium* with a water-supply has already been described (Gregory, 1938). In essence it consisted in attaching the petiole of the leaf to a potometer through tapered glass tubing. The petiole of the leaf was enclosed by two short sleeves of valve-rubber tubing separated by a convenient distance. The lower length of tubing was pushed into the tapered glass tube and a watertight seal thus ensured. Surrounding the tapered end of the potometer was a glass cup. When interruption of the water-supply was desired the cup was half-filled with molten agar on the point of solidification, the petiole was removed from the potometer and immersed in the agar. To re-establish the water-supply to the leaf the surface of the agar was flooded with water and the petiole cut under water between the two rubber sleeves with a portion of a razor blade. The petiole with the upper rubber sleeve was then inserted into the tapered end of the potometer. Each of these operations occupied only a few seconds. The general set-up is shown in Fig. 1, although only one of the rubber sleeves is shown in this figure. Although the lamina of the leaf as shown was enclosed within a chamber, the operations were carried out on the petiole projecting from the base of the leaf chamber by moving the potometer.

In experiments with the wheat leaf the arrangement described needed to be modified. The wheat leaf has no petiole but is inserted on a cylindrical sheath clasping the stem. The leaf sheath was removed from the stem and it now constituted a split cylinder open at both ends. To make contact with a potometer it was necessary to ensure a watertight joint with both the inner and outer surfaces of the sheath. This was achieved by inserting into the sheath *t* a glass rod of suitable diameter covered with valve tubing *s* (Fig. 3). A rubber sleeve *u* was then placed on the outside of the sheath, and this provided a watertight joint when inserted into the tapered end of the potometer *v*. In choosing the glass rod it was essential to select one of such a size that the open edges of the sheath did not overlap. The method described above was used for interrupting the water-supply, but the use of two rubber sleeves was not possible. Instead a length of the leaf sheath was allowed to project beyond the watertight joint; this was sufficiently long to enable a portion to be removed above the level of the agar and the remainder to be reinserted into the potometer. Of course the projection of this length of sheath beyond the watertight joint required that the minimum diameter of the tapered tube should be only slightly less than that necessary for the joint to be established.

4. The air stream

(a) The rate of transpiration was determined by collecting the water vapour given off by the leaf in U-tubes containing phosphorus pentoxide. A diagram of the general arrangement as first used by Pearse is shown in Fig. 4. A current of dry air was drawn through the leaf chamber L. It was thoroughly dried by passage through the calcium chloride tower C_1 and thence through the two U-tubes P_1 and P_2 containing phosphorus pentoxide; after passing over the

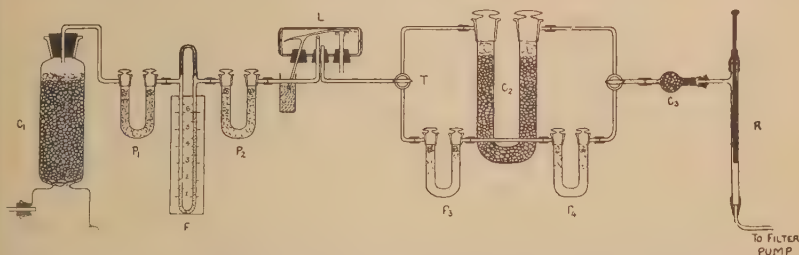


FIG. 4. Layout of apparatus as used by Pearse. C_1 , C_2 , C_3 , calcium chloride contained in drying tower, U-tube, and bulbous tube respectively; P_1 - P_5 , U-tubes of phosphorus pentoxide; F, flow-meter; L, leaf chamber; T, tap; R, variable resistance.

leaf, the transpired water present was absorbed by phosphorus pentoxide contained in two further U-tubes, P_3 and P_4 . When the transpiration rate was not being measured the air stream was diverted through the calcium chloride tube C_2 by turning the three-way tap T, and so any accumulation of vapour in the leaf chamber was prevented. By means of the flow-meter F and the variable capillary resistance R (Gregory, 1933), the rate of air flow was kept constant throughout the experiment. A filter pump was used to maintain the air flow, and the pressure was kept fairly constant by a Mariotte bottle.

The whole apparatus was enclosed in a constant-temperature chamber maintained within 1°C . by means of a capsule thermo-regulator. Heating was effected by wire resistance mats distributed over the floor of the chamber below a grid on which the apparatus stood. The chamber was provided with a movable front of glass through which the manometer could be observed. The roof was also of glass; above it there was a 100-watt lamp screened with running water and distant about 4 in. A temperature of 25°C . was used throughout.

(b) In Spencer's experiments the arrangement was quite similar, except that adjustable needle valves were used in place of the variable resistance. The resistance of the large calcium chloride tube used to by-pass the phosphorus pentoxide tubes was made equal to the latter, so the rate of flow was maintained constant throughout. A constant-temperature chamber was again used to enclose the apparatus. A 150-watt lamp was employed as a source of light; it was screened with running water and placed 10 cm. above the leaf chamber, providing illumination of 1,000 f.c. Most experiments were carried out at a temperature of 18.0 - 18.5°C . and with a flow rate of 38 or 47 l./hr.

(c) An essentially similar arrangement was used by Milthorpe for his early experiments. However, the whole apparatus was kept in a constant-temperature room maintained at 25°C . The chamber was illuminated by a 100-watt lamp, screened with running water, and placed 10 in. above the chamber. This provided an intensity of 200 f.c. at the leaf surface. It was found expedient to reduce the resistance to flow in the system by using a mixture of dry silica gel and phosphorus pentoxide in the absorbing tubes, thus giving smaller pressure gradients for the same rate of flow. With the flow rate of 16 l. hr. used throughout (linear velocity of 3.6 cm./sec.), the air within the chamber was reduced to a pressure of 13 cm. water below atmospheric.

In the later experiments the experimental system was greatly modified to that shown diagrammatically in Fig. 5. These experiments, which will be reported later in this series, were designed to investigate the effects of CO_2 concentration, humidity, rate of air flow, and light intensity on stomatal aperture and transpiration. Any desired relative humidity or CO_2 concentration (below 0.03 per cent.) of the air stream at a particular rate of flow was secured by mixing dry and saturated streams of normal and CO_2 -free air in the required proportions. The rate of flow of the various streams was controlled by needle valves and measured by flow-meters. The air was delivered into the system from a cylinder of compressed air and withdrawn by a suction pump, the resistances being so balanced that the pressure within the chamber was always atmospheric at all rates of flow.

Two streams of air were delivered from the cylinder of compressed air into 10-litre jars (A_1 and A_2). These facilitated the maintenance of an even positive pressure at entry into the system. The magnitude of this pressure was shown by the mercury manometers (M_1 and M_2). One stream (A_1) was scrubbed free of CO_2 by passing it through a bead tower (B_1) containing N. NaOH and then through towers containing soda-lime (D_{1-3}). It then bifurcated for passage either through a tower of P_2O_5 (D_4) or through a bead tower (B_2) containing water which had previously been boiled to displace all dissolved air. The normal air stream (A_2) passed directly through P_2O_5 (D_5) or through water (B_3). The rates of flow of the four streams were adjusted by needle valves (N) and measured by flow-meters (F); they were then mixed and passed through the chamber (c). The positive pressure was balanced by a pull provided by a suction pump at the far end of the system, so that a manometer (M_3) containing paraffin placed adjacent to the chamber indicated atmospheric pressure. A Mariotte bottle (E) was used to maintain an even pressure from the suction pump, and the rate of flow was controlled by the adjacent needle valve (N). A tube of silica gel placed above the Mariotte bottle prevented diffusion of water vapour back into the system. The water vapour in the air stream on leaving the chamber was absorbed in a mixture of P_2O_5 and silica gel contained in the U-tube (u).

In the diagram of Fig. 5 the taps are in the position for air (dry air of 0.01 per cent. CO_2 concentration) flowing through the system during measurement of the transpiration rate. At the end of the transpiration period the taps T_3 and

T_5 were turned through 90° and the air stream diverted through the U-tube (G) containing silica gel. The resistance of this circuit was made equal to that through the U-tube (U) containing P_2O_5 by means of the adjacent needle valve (N). A porometer reading was then taken, the flow of air through the chamber being stopped by turning the taps T_1 and T_6 through 180° . The lower porometer cups were then placed in position and connected through the

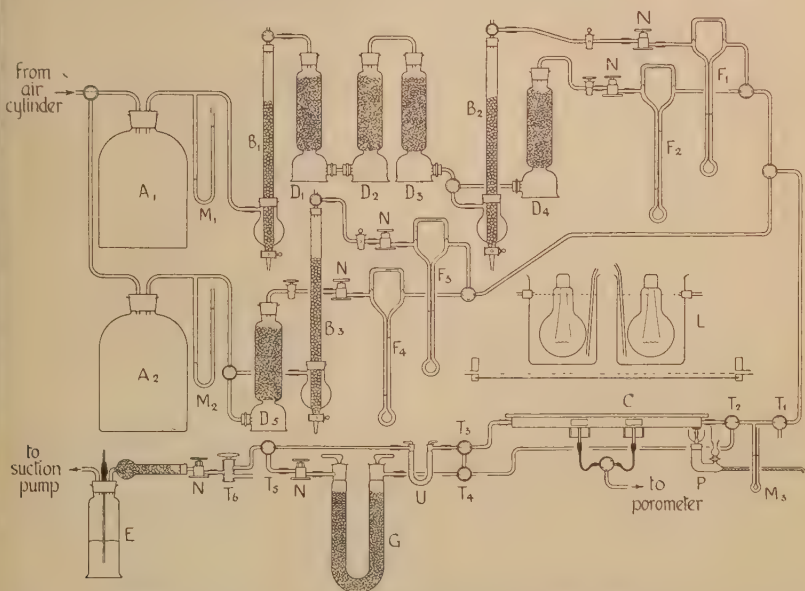


FIG. 5. Arrangement of apparatus as used by Milthorpe in later experiments with wheat. For explanation, see text.

resistance porometer to an aspirator. The air which was drawn through the leaf entered the system through the tap T_1 and was thus of the same composition as was used during the transpiration readings. On completion of the porometer determinations the connexion to the porometer was stopped, the lower porometer cups withdrawn, and the air flow restarted, the air stream being diverted through the by-pass (G) to sweep out accumulated water vapour. Meanwhile the P_2O_5 tube (U) was removed for weighing and another placed in position. At the beginning of the next transpiration period the taps T_3 and T_5 were turned back to their original position and the position of the meniscus in the potometer (P) noted. By turning the taps T_2 and T_4 the chamber could be by-passed and the water content of the air entering the chamber determined. This was necessary when humid air streams were being used.

In this arrangement light was provided by two 300-watt lamps (L) arranged to give as even illumination as was possible along the leaf chamber. Both

lamps were screened by running water, and a further running-water screen below removed radiant heat which escaped the first screen. The distance of the lamps above the chamber could be adjusted to provide a range of intensity from 250 ± 10 to $1,000 \pm 80$ f.c. The use of a counterweight facilitated the adjustment of the lamps and screens.

The flow-meters used in these experiments were of the usual capillary design and were calibrated by Barr's (1934) method.

In the experiments of Pearse and Spencer and the early experiments of Milthorpe air temperature only was measured. This was read on a thermometer, the bulb of which projected into the base of the leaf chamber and was protected from direct radiation by the leaf and a piece of metal foil. In the later experiments of Milthorpe thermo-couples were inserted for measuring both leaf and air temperatures. These were constructed from 48-gauge copper and 40 gauge constantan wires; the couples used for measuring leaf temperatures were soldered to a piece of silver foil (approximately 1.5×1.0 mm.) which pressed against the leaf when in position. The couples for measuring air temperatures were placed in the chamber below the level of the leaf and were protected from direct radiation by a horizontal cylinder of aluminium foil. Both leaf and air temperature couples were placed at positions corresponding to one-quarter and three-quarters of the length of the chamber from the air inlet. The other junctions of the thermo-couples were kept in glass tubes suspended in a thermos flask of water which was maintained at room temperature. The temperature of the flask was read from a thermometer therein to the nearest 0.02° C. The temperature differences were measured on a Cambridge Spot galvanometer; this provided accuracy to 0.015° C. Each couple was calibrated separately before use by measuring the deflexion at known temperature differences.

PROCEDURE OF AN EXPERIMENT

(With detachable porometer cups)

A selected leaf was cut with a clean razor blade from a plant which had been previously well watered. This operation was performed under water with the aid of a water-filled paper funnel held around the base of the petiole. The leaf was then prepared for attachment to the potometer, and with a drop of water still adhering to the cut end of the petiole (or leaf sheath in the case of wheat), the latter was quickly passed through the hole in the chamber base and thence to a vessel of water placed to receive it. The greased rubber stopper holding the leaf in the chamber was placed in position. The lamina was adjusted so that a suitable portion free from large veins was immediately above the porometer cup, which had been previously pushed down. The lid was fixed firmly in position and the upper porometer cup was raised until its washer just made an airtight connexion with the lamina, holding it gently against the glass lid or perspex frame. The plate supporting the upper porometer cup was then locked firmly in position. After testing the porometer to ensure

absence of leaks, the lower cup was drawn down into its recess. The petiole (or leaf sheath) was then fitted to the potometer and the whole system tested for leaks. The air stream was then started, the leaf being left in the chamber, with the light on, the lower porometer cup withdrawn, and the air stream flowing at the constant experimental rate, for a period of 2 hours before beginning experimental readings.

In the experiments with wheat the absorption and loss of water were usually determined over 8-minute periods, with intervals of 2 minutes, during which the air stream was stopped, the lower porometer cup placed in position, and the stomatal resistance measured. The air temperature of the chamber was also recorded. The lower cup was then withdrawn, and the air stream started, being diverted through the silica gel by-pass for at least 30 seconds before transpiration and absorption measurements were resumed. In the experiments with *Pelargonium* transpiration was determined over 15-minute periods with intervals of 10 minutes between them.

At the beginning of the experiment transpiration rates were usually determined over two or three periods before submitting the leaf to a cycle of water-content changes in the manner already described. The experiment was terminated when post-wilting equilibrium had been established. This procedure has been modified in certain experiments, as will be described in the appropriate section.

At the end of each experiment the leaf was removed from the chamber. The area of leaf over the porometer cup was determined by measuring the ring (or rectangle) of grease left on the lamina; in the wheat experiments this portion was then removed and placed in absolute alcohol for stomatal counts at a later date. Finally the leaf area was measured and the water content determined. From the determinations of net gain or loss of water during each transpiration period and of the final water content, the water content at the end of each period was calculated. During the intervals for porometer readings between transpiration periods values for water loss and uptake were interpolated between the respective estimations for the adjacent periods. This procedure assumes that the falls in transpiration and absorption rates during these short intervals of still air were proportional. Consideration of actual determinations for some of the worst cases shows that the errors involved in this assumption are negligible.

SUMMARY

Apparatus and experimental techniques are discussed for use in the investigation of transpiration rate of leaves as controlled by stomatal aperture and leaf water content.

The leaf chambers used and the methods adopted for the estimation of the water transpired are described.

The designs of the porometer cups used for the different types of leaves (*Pelargonium* and wheat) employed in the work are described. To obviate the difficulty that stomata within the cup behave abnormally, the design employed

makes possible a removal of the cup from the leaf except during the short periods required to estimate stomatal resistance to air flow at intervals during the course of an experiment.

In these experiments the water content of the leaf is changed at will by interrupting the water-supply and re-establishing it to permit recovery from wilting. The methods used to carry out this cycle of operations are fully dealt with. Determinations of the transpiration and absorption rates during the experiment and of the final leaf water content make it possible to follow changes in leaf water content throughout the experiment.

An account is given of the methods used for varying the speed of flow, the humidity, and the CO_2 concentration of the air streams.

ACKNOWLEDGEMENTS

We are grateful to Mr. W. Shaw and Mr. F. S. Smith who made the later leaf chambers described in the text, to Mr. D. C. Spanner who kindly constructed the thermo-couples, and to Mr. R. V. Martin for assistance in assembling the apparatus and for preparing the diagrams.

One of us (F. L. M.) held a Farrer Memorial Scholarship during the period of these investigations.

LITERATURE CITED

- BARR, G. (1934). Two designs of flowmeter and a method of calibration. *J. Sci. Instr.* **11**, 321-4.
- BROWN, H. T., and ESCOMBE, F. (1900). Static diffusion of gases and liquids in relation to the assimilation of carbon and translocation in plants. *Phil. Trans. Roy. Soc., B*, **193**, 223-91.
- BURGERSTEIN, A. (1920). *Die Transpiration der Pflanzen*, ii. Jena.
- GREGORY, F. G. (1933). A simple device for regulating gas flow. *Ann. Bot.* **47**, 427-8.
- (1938). A convenient method for attaching potometers and an example of its use in measuring the uptake of water by leaves during recovery from wilting. *Ibid.*, N.S., **2**, 253-5.
- and PEARSE, H. L. (1934). The resistance porometer and its application to the study of stomatal movement. *Proc. Roy. Soc., B*, **114**, 477-93.
- HEATH, O. V. S. (1939). Experimental studies of the relation between carbon assimilation and stomatal movement. I. Apparatus and technique. *Ann. Bot.*, N.S., **3**, 469-96.
- (1941). II. The use of the resistance porometer in estimating stomatal aperture and diffusive resistance. Part 1. A critical study of the resistance porometer. With an Appendix by H. L. Penman. *Ibid.*, N.S., **5**, 455-500.
- KNIGHT, R. C. (1917a). Recent work on transpiration. *New Phytol.* **16**, 127-39.
- (1917b). The interrelations of stomatal aperture, leaf water-content, and transpiration rate. *Ann. Bot.* **31**, 221-40.
- (1922). Further observations on the transpiration, stomata, leaf water-content, and wilting of plants. *Ibid.* **36**, 361-83.
- MAXIMOV, N. A. (1929). *The plant in relation to water*. Allen & Unwin, London.
- NEWTON, R. G. (1936). *The investigation of the relation between rates of assimilation and stomatal aperture in leaves*. Ph.D. Thesis, University of London.
- SCARTH, G. W. (1927). Stomatal movement: its regulation and regulatory rôle. A review. *Protoplasma*, **2**, 498-511.
- SEYBOLD, A. (1929). *Die physikalische Komponente der pflanzlichen Transpiration*. Berlin.
- STÄLFELT, M. G. (1929). Pulsierende Blattgewebe. *Planta*, **7**, 720-34.
- (1932). Der stomatäre Regulator in der pflanzlichen Transpiration. *Ibid.* **17**, 22-85.

Experimental Studies of the Factors Controlling Transpiration

II. THE RELATION BETWEEN TRANSPIRATION RATE AND LEAF WATER CONTENT¹

F. G. GREGORY, F. L. MILTHORPE, H. L. PEARSE, AND
THE LATE H. J. SPENCER

*From the Research Institute of Plant Physiology, Imperial College of
Science and Technology, London*

INTRODUCTION

THE empirical data accumulated by Livingston and his co-workers (Livingston, 1906; Livingston and Brown, 1912; Shreve, 1914), in which the transpiration losses of plants were compared with the water losses from atmometers (relative transpiration), led to the belief that internal factors played a considerable part in the control of transpiration. The speculations on the nature of this factor culminated in Livingston and Brown's (1912) hypothesis of 'incipient drying', in which a major role in determining the rate of water loss was attributed to the degree of imbibition of the cell wall. In essence, this hypothesis supposed that the cell wall may be regarded as a series of capillaries normally filled with water but which in air of sufficient evaporating power became partially depleted so that the menisci of the water surfaces in the capillaries retreated into the wall. This effect introduced two sources of resistance to diffusion: (i) the extra length of diffusion path to the exterior; and (ii) the increased curvature of the water surface with the consequent decrease in vapour pressure.

It should be remarked that the nature of the capillary spaces postulated by Livingston and Brown was not discussed in any detail, but undoubtedly spaces of this kind do exist between the cellulose micellae. Their presence has been confirmed and their dimensions estimated by Frey-Wyssling using the technique of depositing crystals of gold and silver in the wall and submitting to X-ray analyses (Frey-Wyssling, 1937*a, b*). In parenchyma cells these spaces run predominantly parallel with the cell surface and evaporation into the intercellular spaces will be determined by the frequency and dimensions of spaces between the micellae across the wall. Further, later work on the nature of the cellulose wall indicates that it is much more complex than was originally envisaged. Pectin components are invariably present and waxy substances are not only present as cuticle but are also incorporated into the wall structurally (Hess *et al.*, 1936; Gundermann *et al.*, 1937; K. and M. Wuhrmann-Meyer,

¹ This paper includes work embodied in theses submitted to the University of London for degrees of Ph.D. by H. L. P. (1936), H. J. S. (1938), and F. L. M. (1948). The work carried out by F. L. M. was done during the tenure of a Farrer Memorial Scholarship.

1939). It has been shown that the cell walls abutting on the intercellular spaces are non-wettable, due to the presence of substances cuticular in nature (Ursprung, 1925; Bangham and Lewis, 1937; Frey-Wyssling and Hausermann, 1941; Lewis, 1945). The loss of water from the cell cannot be regarded as a simple phenomenon as the evaporating surface must exist somewhere within the cell-wall layers and passage of the liquid phase to this surface can take place through only a portion of the total cell-wall material. In later papers of this series evidence will be presented to show that changes in the permeability of the cell to water occur as a result of reduction in water content so that it appears that the presence of these waxy layers may play a part in controlling transpiration. That this is so in cuticular transpiration is a commonplace.

The direct effect of the reduction in vapour pressure due to the increased concentration of the vacuole contents when water is lost can be neglected owing to the small magnitude of the reduction in vapour pressure. Experiments performed to demonstrate considerable changes in vapour pressure over wilted leaves (Thut, 1939) must be dismissed as faulty.

The whole question of 'incipient drying' as postulated by Livingston was thoroughly experimentally investigated by Knight (1917, 1922). His classical experiment is so well known that it need not be redescribed here. It is necessary, however, to re-examine and reassess his experimental data, since it has been held to prove the view that water content is a major factor in controlling transpiration loss. Knight's experiment purported to show that leafy shoots which had originally been kept under constant conditions of air flow, light, and temperature increased their water content when the air stream was stopped, and that on restarting the air stream the transpiration rate rose to a higher value than previously with the same air flow. The striking innovation in this experiment was the investigation of stomatal behaviour during the cycle of changes by means of an attached porometer cup, and it was found that the stomata remained unchanged in aperture throughout the experiment. It is now known that the behaviour of stomata within such an attached porometer cup for various reasons is not representative of the exposed stomata of the leaf (Heath and Williams, 1948; Heath, 1948), and therefore Knight's assumption that the stomata were not affected cannot be accepted unconditionally. The figures in Table I are reproduced from Knight's experimental data (1917, p. 236, Table IV) with the addition of the amounts of water transpired and absorbed and the total water change in the shoot. The values for stomatal aperture are omitted as they remained fairly constant. It will be seen that during the period 3.30–4.0 p.m., when the air current was stopped, the water deficit decreased from 356 mg. to 273 mg. On restarting the fan the transpiration rate rose to a higher value than that found during the period 1.0–3.30 p.m. prior to the stoppage, while the water deficit increased from 273 to 348 mg. This water deficit was comparable with that existing between 1.30 and 3.30 p.m., but then the transpiration rates were considerably lower. Moreover, the water deficit further increased from 4.30 to 5.30 p.m., and although a fall in transpiration rate accompanied this increase, the transpiration rate did not

TABLE I

Changes in transpiration rate in relation to water deficit as found by Knight (1917). No air movement during the period 3.30-4.0 p.m.

Half-hour periods ending	Amount of water transpired (mg.) (T)	Amount of water absorbed (mg.) (A)	T-A	Water deficit at end of period
12.30 p.m.	518	330	-188	-188
1.0 "	440	390	-50	-238
1.30 "	395	330	-65	-303
2.0 "	405	410	+5	-298
2.30 "	400	380	-20	-318
3.0 "	402	400	-2	-320
3.30 "	416	380	-36	-356
4.0 "	337	420	+83	-273
4.30 "	465	390	-75	-348
5.0 "	447	420	-27	-375
5.30 "	438	400	-38	-413

fall to values as low as those recorded prior to the period of still air when the water deficit was much smaller. It is therefore seen that the increase in transpiration occurring after the period of still air is considerably greater than can be accounted for by the change in water content alone. Similar results are found in Henderson's (1926) experiments.

It would appear therefore that in Knight's experiment water content was not the only factor concerned. It may be pointed out that a whole shoot was used in this experiment. On stoppage of the air current, water would be absorbed by the tissues of the stem owing to the suction pressure transmitted from the leaves. In this experiment the changes in the water content of the leaves themselves were therefore uncertain. The results to be reported in this paper do not suffer from the disadvantage noted above. Leaves alone were under investigation and their water content at all stages was known.

In the course of his work on leaves of *Eupatorium adenophorum* Knight (1922) drew attention to the fact that visible wilting corresponded with a change in water content of 1.5 per cent. of the fresh weight. This apparent small change of water content attending wilting has been subject to much comment. It is, however, very misleading, as the percentage change in fresh weight is the change in the ratio of water in the leaf to the total weight. If W represents the water in the leaf and D the dry weight, the percentage water content will be $100W/(W+D)$. Since W occurs both in the numerator and in the denominator and D is a constant, the ratio will be insensitive to changes of W . It therefore provides no direct measure of changes in the actual water content of the leaf. This may be illustrated by data obtained by Pearse for a *Pelargonium* leaf allowed to wilt in a stream of dry air (Table II). It will be noted that in the first 25 minutes after detaching the leaf the change in percentage water content had amounted to less than 1 per cent., whereas by this time 7.3 per cent. of the original water in the leaf had been lost. By the

TABLE II

Data showing changes in the percentage water content on a fresh weight basis, and the concurrent changes in the water present in the leaf expressed as a percentage of the total water at the beginning of the experiment of a Pelargonium leaf allowed to wilt

Time	Water content (% fresh wt.)	Percentage of total water remaining in leaf
4.20 p.m.	86.04	100.00
4.45 "	85.10	92.68
5.10 "	84.18	86.34
5.35 "	83.41	81.60
6.00 "	83.01	79.26
6.25 "	82.77	77.98
6.50 "	82.60	77.08
7.10 "	82.48	76.42

end of the experiment the leaf was completely wilted and, although the percentage water content had fallen by only 3.5 per cent., nearly a quarter of the total water originally present in the leaf had been lost. Throughout the present experiments the fresh and dry weights of the leaf at the end of each experiment were ascertained, and the data collected on water uptake and loss enabled the changes in the absolute water content of the leaf to be calculated. The apparatus and methods used in these experiments have been described in Part I of this series (Gregory *et al.*, 1950).

WATER LOSS ELIMINATING STOMATAL CONTROL

To eliminate entirely the effects of stomatal control, the method originally used by Darwin (1914) and Henderson (1926) was adopted. The leaves were covered with a layer of vaseline to occlude the stomata and the intercellular spaces opened directly to the air by a series of cuts in the lamina between the main veins.

Data of representative experiments of this kind performed by Pearse on *Pelargonium* are presented in Fig. 1. The curves marked 8, 9, and 10 refer to vaselined and slit leaves, while curves 6 and 7, which present data for entire unvaselined leaves, have been inserted for comparison. In these experiments 6 and 7 the leaves were fitted in the chamber while attached to the plant, the petiole was then cut, the cut end blocked with vaseline, and the leaves allowed to wilt in a stream of dry air. In the other experiments 8, 9, and 10 the leaves were cut off the plant with the petioles under water and were provided with a water-supply while being vaselined and slit. They were transferred to the chamber with the cut end inserted in a potometer in the usual way. The water-supply was broken by blocking with agar as previously described. The data in Fig. 1 show the transpiration in g./m.²/hr. from the time of removing the water-supply.

The data show that in both slit and normal leaves the fall in water content

during the first stage of wilting had no effect on the rate of water loss, and in all experiments transpiration did not begin to decrease until 10–12 per cent. of the total water in the leaf had been lost. After this time, both with slit and normal leaves, the transpiration rate fell rapidly with decreasing water content.

Similar experiments were carried out by Milthorpe with wheat leaves. Results of three experiments of this kind are shown in Fig. 2. In the case of

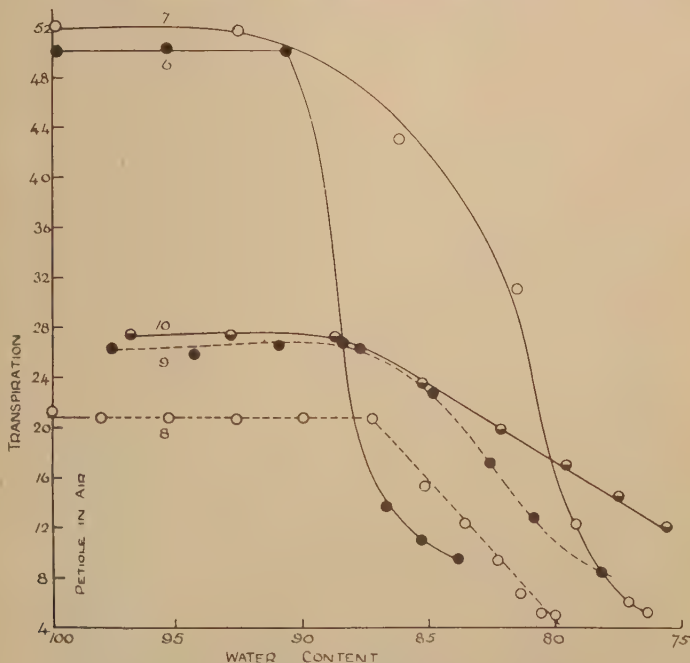


FIG. 1. Relation between transpiration rate ($\text{g./m.}^2/\text{hr.}$) and leaf water content (% original amount) in *Pelargonium*. Curves 6 and 7, stomatal control present; curves 8, 9, and 10, stomatal control eliminated.

the wheat leaf, the decrease in rate of transpiration occurred with a smaller deficit in water content than in *Pelargonium*. Nevertheless the same phenomenon appeared. It is probable that no change in rate of water loss occurs until 5 per cent. of the total water has been lost.

The actual rate of water loss for slit leaves of both plants was only half that of leaves with functional stomata, indicating the more efficient evaporation through the normal stomatal channels than through the open cuts. The fall in rate of water loss in the slashed leaves may in part be due to drying of the cut edges of the slits and partial closure of the spaces by collapse of the cells. A general reduction in the dimensions of the intercellular spaces by loss of turgor of the leaf may certainly be postulated. Furthermore, at the time the rate first begins to fall the water content of the tissues near to the slits will

certainly be appreciably lower than the average calculated for the leaf as a whole. It is therefore likely that the values of 5 per cent. for wheat and 10–12 per cent. for *Pelargonium* as the amount of water which may be lost before the rate of loss is affected are underestimated.

WATER LOSS WITH STOMATAL CONTROL

In these experiments the aim has been to change at will the water content of the leaf by temporarily stopping the water-supply and later re-establishing

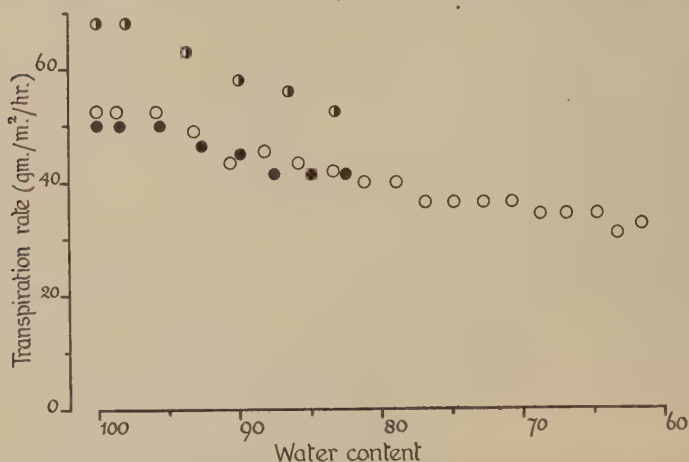


FIG. 2. Relation between transpiration rate (g./m.²/hr.) and leaf water content (% original amount) during its reduction on cessation of water-supply in three separate leaves of wheat, with stomatal control eliminated.

it. The necessary means of achieving this end have already been described in Part I of this series (Gregory *et al.*, 1950). During the phase of falling water content the cut end of the petiole (*Pelargonium*) or leaf sheath (wheat) was blocked with agar, and the rising phase was induced by recutting under water and reinserting into the potometer. The water loss was followed throughout the cycle by absorption of the water vapour and weighing over periods of 15 minutes in *Pelargonium* and 8 minutes in wheat. Water absorption was measured during the preliminary period before interrupting the water-supply and again immediately after replacing the recut petiole into the potometer. Stomatal resistance was measured with a detachable porometer cup at intervals of 25 minutes in *Pelargonium* and 10 minutes in wheat throughout the cycle.

Replacing the petiole into the potometer occupied some 30 seconds, so that the uptake rate during the first minute of recovery was uncertain. A value was obtained by extrapolation from successive minute readings after the first. The re-entry of water into the leaf was very rapid at first owing to the increased suction pressure of the leaf tissue, and the course of uptake in *Pelargonium* during recovery from wilting has already been published (Gregory, 1938).

After renewing the water-supply, water content reached equilibrium within 15–25 minutes in the *Pelargonium* leaf and within 20–30 minutes in the wheat leaf, so that by the end of the first transpiration period with *Pelargonium* and by the end of the third with wheat, uptake had fallen to the same rate as transpiration.

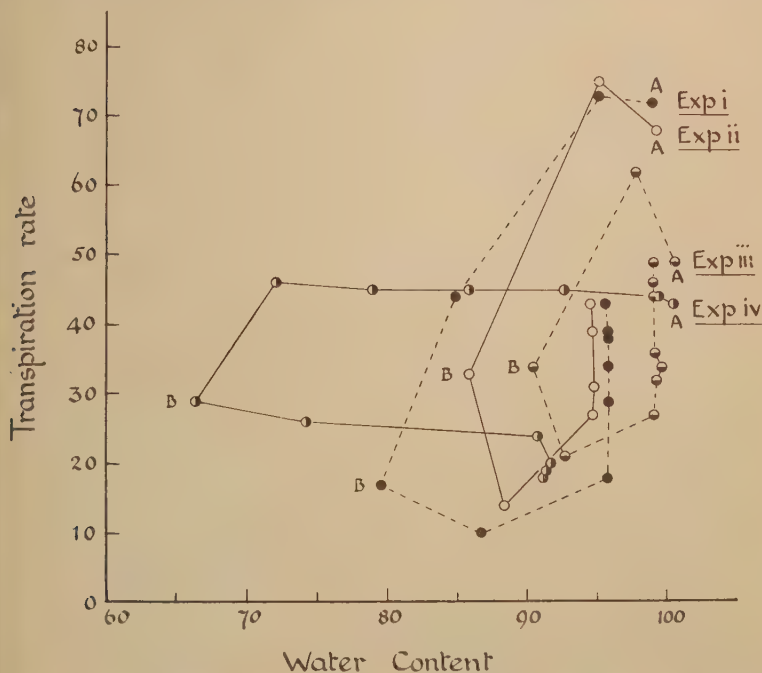


FIG. 3. Relation between transpiration rate (g./m.²/hr.) and mean leaf water content (% original amount) during induced cycles of changing water content in four separate leaves of *Pelargonium*, with stomatal control present. Water-supply to leaf stopped at end of period marked A and restored at end of period marked B in each experiment.

(i) Experiments with *Pelargonium*

To indicate the kind of relationship obtained between transpiration and water content in the *Pelargonium* leaf, the results of four experiments carried out by Spencer are presented in Fig. 3. The full data will be considered later when the interaction of all factors concerned is dealt with. It suffices here to say that the experiments were carried out in dry air at a temperature of 18.5° C. with a flow rate of 47 l./hr. and a light intensity of 600 f.c.

In Fig. 3 the transpiration rate in g./m.²/hr. is plotted against the mean water content for each successive period. At the end of the period marked A the water-supply was interrupted and at the end of the period marked B the water-supply was renewed to the leaf. The water-content changes are given as

percentages of the water content at the time at which the water-supply was cut off. It will be noted that the degree of water depletion at the time of renewal of the water-supply varied greatly with the different experiments. This was achieved by varying the time over which wilting was allowed to proceed. Each point of the curves represents a period of 15 minutes separated by an interval of 10 minutes over which transpiration was not measured; so the duration of wilting may be directly ascertained from the figure.

In Expts. i, ii, and iii the transpiration rate increased during the first 15 minutes of wilting, but this effect was not seen in Expt. iv. This increase in transpiration was associated with the preliminary opening of the stomata in the early stages of wilting as noted by Darwin and Pertz (1911) and Laidlaw and Knight (1916). This was followed in Expts. i-iii by a rapid fall in transpiration up to the point marked *B*, at which point the water-supply was renewed. In Expt. iv, however, transpiration remained steady for a period of 95 minutes, by which time the water content had fallen by nearly 30 per cent. The transpiration rate then fell. In the first period of 15 minutes after the point marked *B*, in all cases, the water content rose rapidly but transpiration continued to fall. In Expts. i-iii final water equilibrium had been reached within 15 minutes, and it is seen that the degree of recovery approached completion (100 per cent.) only in Expt. iii, in which wilting was continued for 40 minutes only and in which the water content never fell below 90 per cent. In Expts. i and ii wilting was more severe and recovery of water content less complete. The most striking result seen in this diagram is that after the water content had reached its final equilibrium the transpiration rate continued to rise rapidly with a steady water content over a period of 2 hours. In Expt. iii the transpiration rate returned to its original value, but it did not do so in Expts. i and ii.

In Expt. iv, in which wilting was much more severe, the water content eventually rose to about 92 per cent. of the original value, but the increasing water content was not accompanied by an increase in the transpiration rate, which remained at a low value until the end of the experiment.

(ii) *Experiments with wheat*

In the experiments carried out by Milthorpe using the wheat leaf the procedure was essentially the same. However, in some experiments the leaves were submitted to more than one cycle of wilting and recovery. Here only certain of the results are presented graphically to show that the effects noted by Spencer in *Pelargonium* were also found in wheat; namely, increasing transpiration rates accompanied decreasing water content on wilting, falling transpiration rates occurred as the water content was restored, and the transpiration rate after recovery from wilting continued to rise for a considerable period after the water content had reached equilibrium (Fig. 4).

These points are well illustrated by the data of Fig. 5 which refer to a leaf submitted to four cycles of wilting and recovery. It is here shown that the transpiration rate varied very considerably at any one water content. For

instance, with a water content of 83 per cent. of the original value, transpiration rates ranging from 23 to 70 g./m.²/hr. were found. The partial regression equation of transpiration (T) on log stomatal resistance ($\log R$) and leaf water content (W) in this experiment was found to be

$$T = 46.67 - 13.0368(\log R - 3.079) + 0.2273(W - 86.57).$$

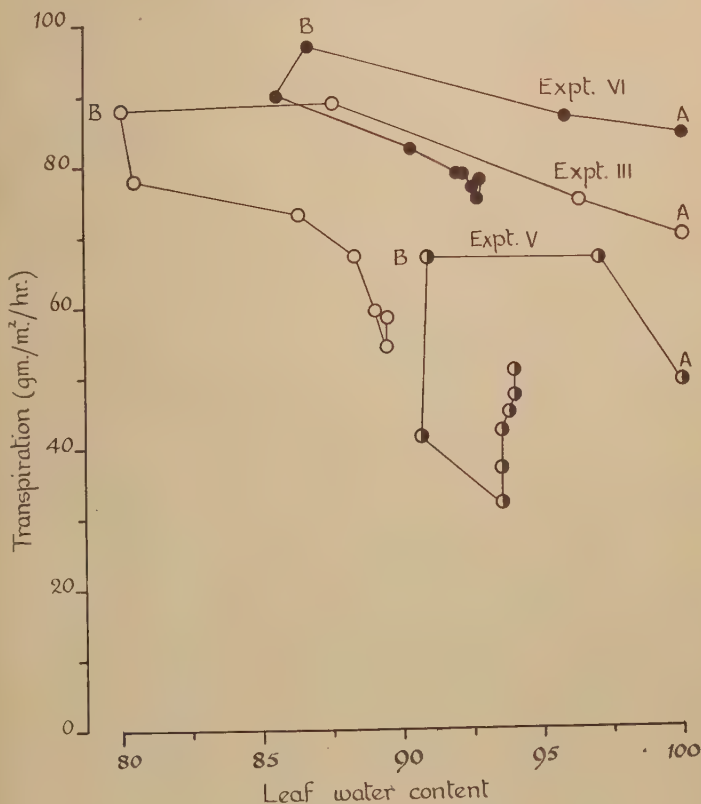


FIG. 4. Relation between transpiration rate (g./m.²/hr.) and mean leaf water content (% original amount) during induced cycles of changing water content in three leaves of wheat, with stomatal control present. Water-supply stopped at end of period marked A and restored at end of period marked B.

The partial regression coefficient of transpiration on leaf water content (0.2273) was not significant ($t = 1.48$, $n = 30$); so even when the effect of stomatal aperture was eliminated, no relation between transpiration rate and leaf water content could be demonstrated.

The relation between transpiration and water content over a whole series of experiments with wheat are summarized in Fig. 6. The water content varied

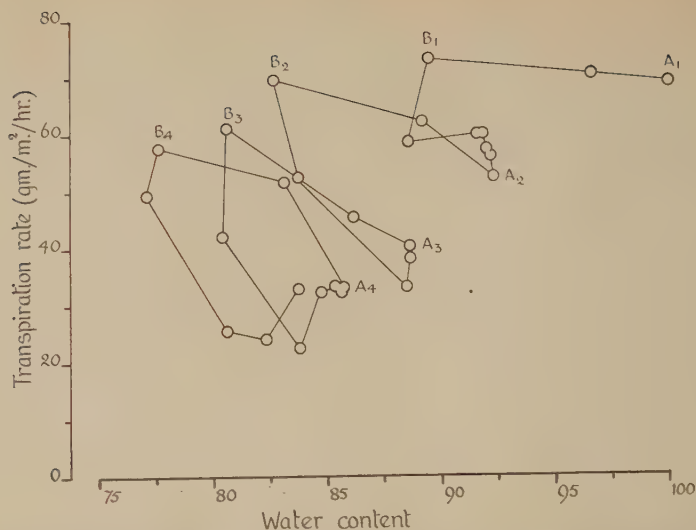


FIG. 5. Relation between transpiration rate ($\text{g./m.}^2/\text{hr.}$) and leaf water content (% original amount) during four successive cycles of changing water content in a wheat leaf, with stomatal control present. Water-supply to leaf stopped at end of periods marked A_1 , A_2 , A_3 , and A_4 , and restored at end of periods marked B_1 , B_2 , B_3 , and B_4 .

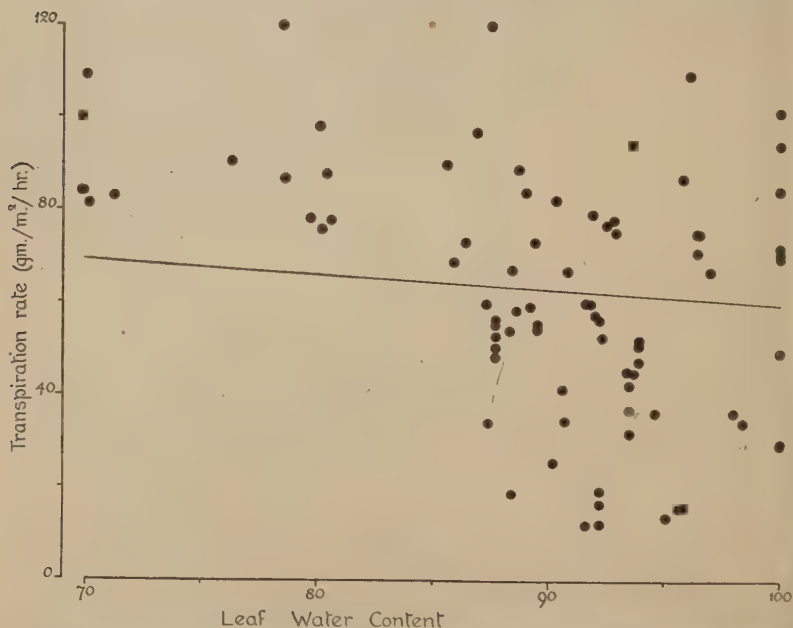


FIG. 6. Correlation between transpiration rate ($\text{g./m.}^2/\text{hr.}$) and leaf water content (% original amount) for 88 determinations obtained from 9 different leaves of wheat.

from 100 to 69 per cent. over the 88 values from 9 experiments entered. The regression equation relating transpiration rate (T) to log stomatal resistance ($\log R$) and relative water content (W) was

$$T = 63.04 - 13.4816(\log R - 1.81) - 0.3274(W - 89.44).$$

Again there was no indication of any relation between transpiration and water content; indeed the partial regression coefficient (-0.3274) indicated a rise in transpiration with falling water content, but this is statistically insignificant ($t = 1.75$, $n = 85$).

The line shown in Fig. 6 indicates the relation between transpiration rate and leaf water content which would be expected if the stomatal aperture was maintained at the mean value of log stomatal resistance (1.181) for these data.

AN EXAMINATION OF KNIGHT'S EXPERIMENT

Earlier in this paper the results of Knight's (1917) classical experiment were reassessed (p. 16), and it was concluded that the increase in transpiration rate recorded in this experiment was greater than could be accounted for by the change in water content alone. It was pointed out that Knight used an attached porometer cup and that it is now known that the behaviour of stomata within such a cup is not representative of those on the exposed portion of the leaf (Heath and Williams, 1948). Consequently the experiment of Knight was repeated, using a detached wheat leaf fitted to a water-supply and employing the porometer cup, previously described (Gregory *et al.*, 1950), which was detached from the leaf between readings. The technique was similar to that used in all these experiments, except that the air stream was stopped for a period, during which time the stomatal behaviour was noted.

One such experiment is shown in Fig. 7. The leaf was in a stream of dry air until the point marked *a*, when the air flow was stopped. During the period of still air (*a-b*) the stomata opened considerably as indicated by falling log R (black circles). When the air flow was restarted at *b*, the transpiration (shown by continuous lines) was higher than prior to the stoppage, but this decreased with time. This fall in the transpiration rate was accompanied by stomatal closure. When the stomata had reached the resistance shown prior to the period of still air, the transpiration rate had returned to its original value. The water content (shown as white circles) had increased by 1 per cent. during the period of still air. This confirms Knight's finding of increased water content during stoppage of air flow, but as it was maintained during the subsequent exposure to moving air and during the period when transpiration rate and stomatal aperture decreased, it cannot be held to influence the transpiration rate. Still-air periods were given at *e-f* and *g-h* after a period of wilting and recovery at *c-e* and similar effects were noted. It thus appears that stomata open wider in still than in moving air. It seems certain that Knight misinterpreted his experiment owing to the fact that the stomata on which measurements were made were always protected in an atmosphere of still air by the

attached cup used and therefore gave no indication of the behaviour of the stomata on the exposed portions of the leaves. The wider opening of stomata in still as compared with moving air probably results from a lower CO_2 concentration of the air. It has recently been shown (Heath, 1948) that the stomata open widely in response to a reduction of the CO_2 concentration normally present in the air, and such a reduction would be expected in the still-air conditions of these experiments as a result of assimilation. The CO_2 concentration would be higher in the chamber with moving air as it would be

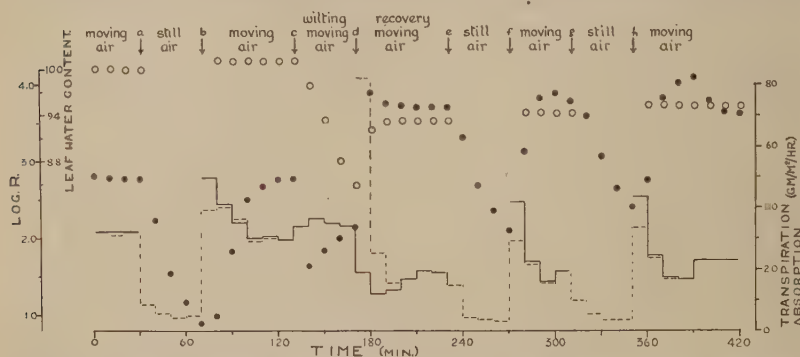


FIG. 7. The effect of periods of still air on transpiration rate (continuous lines), absorption rate (broken lines), log stomatal resistance (log R , black circles), and leaf water content (white circles) of the wheat leaf. Air flow stopped at *a*, *e*, and *g*, and restored to original rate of 16 l./hr. at *b*, *f*, and *h*. Water supply removed at *c* and restored at *d*. (Expt. vii.)

continually renewed to the leaf. It is also known that (in wheat) dry moving air causes closure of the stomata; this effect will be discussed elsewhere at a later date.

DISCUSSION

It has been shown that under the conditions of these experiments transpiration is quite independent of water content. When it is borne in mind that in ecological work performed under natural conditions the plants used may have suffered from partial wilting to an unknown extent, it may at least be doubted whether the relation obtained between transpiration rate and water content in such experiments has much meaning. The changes in water content which often accompany the diurnal changes in evaporating power of the air (Miller, 1917, and others) cannot be accepted as evidence that water content is a regulatory factor in water loss. It is quite obvious that when the evaporating power of the air rises, the maintenance of the transpiration stream against increasing resistance to entry of water into the plant due to factors in the soil and at the root surfaces, demands an increase in the suction pressure of the leaves. This is achieved by a loss of water from the leaf cells. Whether the leaf wilts under these conditions or not depends upon the properties of the cell wall. With very elastic walls the suction pressure of the cells can rise con-

siderably before onset of wilting, which will only begin after the cell wall is completely relaxed; the degree of change in water content at this stage will depend entirely on the elastic properties of the wall.

In this paper, of the factors controlling transpiration rate, only water content has been considered. The part played by the stomata will be dealt with in the next paper of this series. The striking result to which attention has been drawn, that transpiration continues to rise after water equilibrium has been established, is not, however, entirely due to stomatal regulation, and a further factor of permeability change in the cell wall appears probable. Evidence for this statement will be presented in due course.

SUMMARY

Data are presented which show, when stomatal control is eliminated, that wheat leaves may lose 5-6 per cent. and *Pelargonium* leaves 10-12 per cent. of their water without any reduction in the transpiration rate.

Experiments in which *Pelargonium* and wheat leaves, with stomatal control present, were submitted to cycles of changing water content also failed to establish any direct relation between transpiration rate and leaf water content.

It is concluded that leaf water content over the range of 70-100 per cent. of that present in the turgid state has no significant effect in determining the rate of water loss from leaves.

A repetition of Knight's experiment showed that stomata opened in still air and closed in moving air. This was not recorded by Knight, who used a porometer cup permanently attached to the leaf. It is concluded that the higher transpiration rate recorded by Knight after a period of still air was due to wider stomatal aperture and not to the higher leaf water content as suggested by him.

LITERATURE CITED

- BANGHAM, D. H., and LEWIS, F. J. (1937). Wettability of the cellulose walls of the mesophyll in the leaf. *Nature*, **139**, 1107-8.
- DARWIN, F. (1914). The effect of light on the transpiration of leaves. *Proc. Roy. Soc., B*, **87**, 281-99.
- and PERTZ, D. F. M. (1911). On a new method of estimating the aperture of stomata. *Ibid.* **84**, 136-54.
- FREY-WYSSLING, A. (1937a). Röntgenometrische Vermessung der submikroskopischen Räume in Gerüstsubstanzen. *Protoplasma*, **27**, 372-411.
- (1937b). Ultramikroskopische Untersuchung der submikroskopischen Räume in Gerüstsubstanzen. *Ibid.* 563-71.
- and HAUSERMANN, E. (1941). Über die Auskleidung der Mesophyllinterzellularen. *Ber. schweiz. Bot. Ges.* **51**, 430-1.
- GREGORY, F. G. (1938). A convenient method for attaching potometers and an example of its use in measuring the uptake of water by leaves during recovery from wilting. *Ann. Bot.*, N.S. **2**, 253-5.
- MILTHORPE, F. L., PEARSE, H. L., and SPENCER, H. J. (1950). Experimental studies of the factors controlling transpiration. I. Apparatus and experimental technique. *J. Exp. Bot.* **1**, 1-14.
- GUNDERMANN, J., WERGIN, W., and HESS, K. (1937). Über die Natur und das Vorkommen der Primärsubstanz in den Zellwänden der pflanzlichen Gewebe. *Ber. deut. chem. Ges.* **70**, 517-26.

- HEATH, O. V. S. (1948). Control of stomatal movement by a reduction in the normal carbon dioxide content of the air. *Nature*, **161**, 179-81.
- and WILLIAMS, W. T. (1948). Adequacy of the porometer in the investigation of stomatal aperture. *Ibid.* 178-9.
- HENDERSON, F. Y. (1926). On the effect of light and other conditions upon the rate of water-loss from the mesophyll. *Ann. Bot.* **40**, 507-33.
- HESS, K., TROGUS, C., and WERGIN, W. (1936). Untersuchungen über die Bildung der pflanzlichen Zellwand. *Planta*, **25**, 419-37.
- KNIGHT, R. C. (1917). The interrelations of stomatal aperture, leaf water-content, and transpiration rate. *Ann. Bot.* **31**, 221-40.
- (1922). Further observations on the transpiration, stomata, leaf water-content, and wilting of plants. *Ibid.* **36**, 361-83.
- LAIDLAW, C. G. P., and KNIGHT, R. C. (1916). A description of a recording porometer and a note on stomatal behaviour during wilting. *Ibid.* **30**, 47-56.
- LEWIS, F. J. (1945). Physical conditions of the surface of the mesophyll cell walls of the leaf. *Nature*, **156**, 407-9.
- LIVINGSTON, B. E. (1906). The relation of desert plants to soil moisture and to evaporation. *Carnegie Inst. Washington Public. No. 50*.
- and BROWN, W. H. (1912). Relation of the daily march of transpiration to variations in the water content of foliage leaves. *Bot. Gaz.* **53**, 309-30.
- MILLER, E. C. (1917). Daily variation of water and dry matter in the leaves of corn and the sorghums. *J. Agr. Res.* **10**, 11-47.
- SHREVE, E. B. (1914). The daily march of transpiration in a desert perennial. *Carnegie Inst. Washington Public. No. 194*.
- THUT, H. F. (1939). The relative humidity gradient of stomatal transpiration. *Amer. J. Bot.* **26**, 315-19.
- URSPRUNG, A. (1925). Über das Eindringen von Wasser und anderen Flüssigkeiten in Interzellularen. *Beih. Bot. Zentralb.* **41**, 15-40.
- WUHRMANN-MEYER, K. and M. (1939). Über Bau und Entwicklung der Zellwände in der *Avena-Koleoptile*. *Jahrb. f. wiss. Bot.* **87**, 642-78.

Studies in Stomatal Behaviour

V. THE ROLE OF CARBON DIOXIDE IN THE LIGHT RESPONSE OF STOMATA

PART I

Investigation of the Cause of Abnormally Wide Stomatal Opening within Porometer Cups

O. V. S. HEATH

*Research Institute of Plant Physiology, Imperial College of Science and Technology,
London*

	PAGE
INTRODUCTION	29
MATERIALS AND METHODS	32
EXPERIMENTAL RESULTS	
I. Direct microscopical observations on the living leaf	32
II. Preliminary experiments with the infiltration technique	35
III. Porometer experiments	37
IV. Further experiments with the infiltration technique	40
V. Preliminary discussion	44
VI. Further porometer experiments to test the CO ₂ hypothesis	46
VII. Further experiments with the infiltration technique to test the CO ₂ hypothesis	52
GENERAL DISCUSSION	
1. The carbon dioxide effect	54
2. Relevance to other investigations with the porometer	56
3. Subsidiary observations	58
4. Biological importance of the carbon dioxide effect and humidity effects	59
5. The importance of equilibrium values in porometer experiments	60
SUMMARY	60
ACKNOWLEDGEMENTS	61
LITERATURE CITED	61

INTRODUCTION

In the original porometer of Darwin and Pertz (1911) a small cup was attached to the leaf surface and left in position throughout an experiment, thus permanently enclosing a portion of the surface. Until recently this method has been followed in all subsequent porometer investigations, with the notable exception of some experiments by Spencer (see below). When the porometer was used for purely qualitative purposes, to show merely if the stomata were opening or closing, the question of whether the stomata within the cup had at all times the same mean apertures as those on the unenclosed portion of the leaf was relatively unimportant; nor was there any great objection to using porometer readings of rate of flow of air through the leaf (Darwin and Pertz, 1911; Knight, 1915) or resistance to such flow (Gregory and Pearse, 1934), uncorrected for the irrelevant internal resistances in the leaf. For the more quantitative investigation of the dynamics of stomatal movement (Gregory and Pearse, 1937; Williams, 1940) calibration of the 'resistance porometer'

in terms of actual stomatal aperture would seem most desirable, while for work on the relation between stomatal movement and carbon assimilation (Maskell, 1928; Heath, 1939) or transpiration (Gregory *et al.*, 1950, 1950a) calibration in terms of stomatal resistance to *diffusion* is urgently needed. For the latter purpose the 'diffusion porometer' of Gregory and Armstrong (1936) may be used to calibrate the much more convenient 'resistance porometer'. For both these calibrations the first essential is to be able to estimate from 'resistance porometer' readings the resistance to viscous flow of air of the stomata within the cup only, thus eliminating the confusing effects of the varying amounts of the resistances due to the intercellular space system and stomata external to the cup which are included in the total resistance measured (Newton, 1936; Heath, 1941). This is made possible by the mathematical theory of porometers elaborated by Penman (1942) and used by the author (Heath, 1941) to estimate the resistance of *Pelargonium* stomata to viscous flow of air. Penman also gives an approximate theory for the diffusion porometer which enables similar estimates to be made of the *diffusive* resistance of stomata within the cup. Hence, the desired calibration of the resistance porometer in terms of diffusive resistance of the stomata alone should be possible.

A great quantity of data had been collected by the author for the above two calibrations, but the conclusions as to stomatal apertures within and outside a permanently attached porometer cup, reached by Heath and Williams (1948) and discussed below, render the data for the attempted calibration in terms of aperture valueless. The data collected for the *relation* between viscous and diffusive resistance may perhaps be of some value, but require further experimental checking and much further work is needed.

It is obvious that in any theory of porometers, such as that of Penman, which is concerned with the distribution of viscous or diffusive flow through the leaf, a necessary assumption is that mean stomatal apertures are the same on all parts of the leaf, or else that they differ in a known manner. The former assumption is implicit in Penman's analysis. Observations by means of Lloyd's (1908) method, made by Newton (1936) for the *Pelargonium* leaf with moderately closed stomata, and a similar isolated observation made by the author, had suggested that stomatal apertures within and outside porometer cups were of similar magnitude. The discovery that stomata on an illuminated leaf of *Pelargonium* in fact open to a very much wider extent where enclosed in a porometer cup than elsewhere on the leaf (Heath and Williams, 1948) only arose recently, out of an investigation by Williams (1947, 1949) of the sensitivity of the stomata to mechanical 'shock' stimuli. A detailed account of some of the steps leading to this discovery has been presented in a previous paper of this series (Williams, 1949). The writer, concurrently with Williams and after consultation with him, carried out a number of experiments which led to the same conclusion and showed that it also applied to the wheat leaf. These experiments are described briefly in the present paper.

The abnormally wide opening, in the light, of enclosed stomata being established beyond all doubt, it became necessary to modify the porometer so

that this no longer occurred. Suitable modifications were outlined in the preliminary note (Heath and Williams, 1948), the most important of these being the use of a detachable cup, or, alternatively, sweeping the cup between readings with air of the same composition as that surrounding the rest of the leaf, and having the area of leaf immediately above the cup uncovered. The use of a cup which is detached from the leaf at all times except when readings are actually being taken is far preferable to the 'sweeping' method; such a detachable cup, which could be used even on the hairy leaves of *Pelargonium*, was employed by Spencer (Spencer, 1938; Gregory *et al.*, 1950) in this Institute for a special purpose and later by Milthorpe (Gregory *et al.*, 1950, 1950a) with wheat. A somewhat simpler form is now in use for *Pelargonium*. It is hoped to discuss the modifications of the porometer, together with some theoretical considerations, in another paper.

The main purpose of the present paper is to give a detailed account of the investigation, already reported briefly (Heath, 1948), which led to the finding that the abnormally wide stomatal opening within porometer cups is due to reduction by photosynthesis of the carbon dioxide content of the enclosed air below the normal 0.03 per cent.

The importance of this sensitivity of the stomata to small changes of carbon dioxide content below the normal level will be considered in the general discussion. That it has not been established previously, in spite of more than fifty years of sporadic investigation of carbon dioxide effects upon stomata, would seem to be because the comparison has almost always been made between either carbon dioxide-free or ordinary air on the one hand, and very high concentrations or even pure carbon dioxide on the other. Thus Darwin (1898) found with his horn hygroscope that stomata remained open in the light in humidified carbon dioxide-free air (dark readings under these conditions were apparently not taken), whereas in pure carbon dioxide they closed slowly. He attributed Schellenberger's finding that the stomata *closed* in carbon dioxide-free air to the drying effect of the 10 per cent. KOH used by the latter to remove the carbon dioxide. Some of Lloyd's (1908) results suggest that in *Verbena ciliata* the stomata can be prevented from closing completely in the dark by carbon dioxide-free air, but that if already closed they will not reopen; in light they open in carbon dioxide-free air. Lloyd himself does not call attention to these indications, which are confirmed by one of the experiments on *Pelargonium* described below. Linsbauer (1916) investigated the effects of carbon dioxide-free air both in light and in the dark and found that removal of carbon dioxide favoured opening under both conditions. He stated that the effect was less pronounced in light owing to the stomata being already partly open. Insufficient weight has perhaps been given to Linsbauer's results because of the very crude technique employed: fragments of leaf were held against moist filter-paper over solid KOH or a strong solution, and Linsbauer himself stated that much trouble was caused by the tendency of the leaf fragments to dry. He found that high concentrations of carbon dioxide caused closure or failure to open in either light or darkness. Stahl (1919) also

found that high carbon dioxide content caused stomatal closure. Scarth (1932) found that in darkness high concentrations of carbon dioxide (10–50 per cent.) caused stomatal closure followed after many hours by opening. The effects of carbon dioxide-free air were apparently not investigated.

MATERIALS AND METHODS

Pelargonium zonale leaves from three sources were used in these investigations: plants of var. 'Paul Crampel', both of 'Clone 5' (Heath, 1941) and also of another strain, grown at Chelsea Physic Garden, and plants grown in the Botany Garden at Bedford College supplied by the courtesy of Dr. W. T. Williams. The latter had much smaller leaves than the two former strains, and the stomata were apparently more responsive.

Wheat leaves of two Australian varieties (viz. var. 'Charter' and var. 'Bencubbin') were also used. These plants were kindly supplied by Dr. F. L. Milthorpe, who grew them in this Institute.

The methods used included direct observation of the living leaf with transmitted light, much as described elsewhere (Heath, 1938), but without the bull's eye and water screen, Lloyd's (1908) method, Williams's (1949) modification of Molisch's (1912) infiltration method, and the 'resistance porometer' (Gregory and Pearse, 1934; Heath, 1941). Details of technique were varied as the investigation proceeded and will be mentioned under the relevant experiments.

The original numbering of the experiments is retained for ease of checking and future reference. All times are given in G.M.T.

EXPERIMENTAL RESULTS

I. *Direct microscopical observations on the living leaf*

These investigations were begun when Dr. W. T. Williams informed the writer that strips taken by Lloyd's (1908) method from the area of leaf enclosed by a porometer cup showed very much wider stomatal apertures than those taken from the rest of the leaf (see Williams, 1949, fig. 3, p. 314). It was deemed essential to discover whether such differences in aperture existed on the living and intact leaf. While Williams attacked the problem by the infiltration method, as described in the paper cited, the writer carried out the following direct microscopical observations.

In these experiments the microscope was used horizontally and the leaf, on a potted plant, was tied back into a vertical position so that its upper (adaxial) surface rested against a slide on the microscope stage. In some cases a rubber band was used to keep the leaf against the stage. In all experiments the lower (abaxial) leaf surface was exposed to diffuse daylight in addition to the other sources of illumination mentioned. The leaf used was always the third fully expanded leaf on a shoot and of 4–4½ in. diameter.

Expt. 35. Pelargonium, Clone 5.

23.9.47. The leaf was set up at 9 a.m. and examined with a 4-mm. apo. objective, corrected for lack of cover glass. No visible apertures could be distinguished among 25 stomata in the field of view after prolonged illumination: first, with light supplied through the condenser from a 12-V. 24-W. lamp, and later of the whole lower surface of the leaf with a 60-W. lamp at 9 in. with white reflector but no cooling screen.

24.9.47. The same leaf was set up and examined with an 8-mm. apo. objective at 10.30 a.m. All stomata appeared firmly closed. The objective was next surrounded with a 2-cm. diam. 'van Tieghem' glass ring dipped in gelatine, coated with luting wax, and used to press the leaf against the microscope slide much as in a normal porometer-cup fitting. The pressure was supplied by rubber bands. Illumination as before failed to produce any visible opening of the stomata.

25.9.47. The same leaf was set up on a porometer cup of the usual type (Heath, 1941) in daylight at 11.0 a.m. Between 11.7 a.m. and 1.30 p.m. the total resistance as estimated fell steadily from 0.60 to 0.076 units, indicating that the stomata were responsive to light, at least when enclosed in a porometer cup. The cup was then removed and strips were taken within and outside the cup area by Lloyd's method. These showed differences of aperture similar to those figured by Williams (1949, Fig. 3, 'Cup A' and 'outside'), but somewhat less extreme. Nearly all stomata on the strips from outside the cup showed some degree of opening though much less than within the cup.

Expt. 36. Pelargonium, Clone 5.

30.9.47. The leaf was set up at 10.30 a.m. and illuminated with a 150-W. lamp, in a running-water screen, 6 in. from the *lower* surface. Examination with the 8-mm. objective at intervals up to 2.0 p.m. failed to show any open stomata. From 2.15 p.m. the *upper* surface of the leaf was similarly illuminated, the microscope condenser being removed to allow of this. Examination at intervals up to 4.30 p.m. again showed no open stomata.

1.10.47. The same leaf was set up, with the *upper* surface illuminated, at 9.30 a.m. Examination with the 8-mm. objective at 11.30 a.m. again gave no evidence of stomatal opening. At 11.45 a.m. strips of lower epidermis were taken by Lloyd's method. These showed many stomata with small and medium openings and only a few completely closed, much as on the strips taken outside the cup area in Expt. 35 on 25.9.47.

At midday a 'dummy porometer cup' surrounding the objective was set up as in Expt. 35 (24.9.47). Illumination of the upper surface as before failed to produce any visible opening of the stomata, which were examined at 1.40 p.m., 2.55 p.m., and 5.45 p.m.

2.10.47. The same leaf was set up on a porometer cup at 9.55 a.m. and illuminated with the 150-W. lamp and screen at 5 in. The resistance fell from 2.22 units at 10.0 a.m. to 0.049 unit at 1.30 p.m. By 2.0 p.m. slight closure had begun (0.057 unit) and by 2.15 p.m. the resistance had risen to 0.20 unit. Clearly the stomata were closing rapidly, presumably owing to a diurnal rhythm; the leaf was quickly removed and the lower surface examined with the 8-mm. objective. Within the cup area many stomata appeared moderately wide open, but outside it no apertures could be seen. Strips of lower epidermis were next taken both from within and

outside the cup area. The latter showed most of the stomata *slightly* open but many closed; from within the cup the apertures appeared somewhat wider than they had been on the living leaf.

Expt. 37. *Pelargonium*, Clone 5.

8.10.47. The leaf was set up at 11.40 a.m. with a 'dummy porometer cup' surrounding the 8-mm. objective. All the stomata in the field of view appeared shut, both at the time of setting up and also at 1.30 p.m., after illumination for $1\frac{1}{2}$ hours with the 150-W. lamp in screen at 5 in. from the *lower* surface of the leaf. At 1.53 p.m., the objective being racked back, the van Tieghem cell was covered with a cover-slip (which was not greased on) and the leaf illuminated as above until 3.40 p.m. On examination one stoma of the many in the field of view then showed a slight opening. It should be noted, however, that this was relatively late in the afternoon. The leaf was left set up overnight with the van Tieghem cell and cover-slip in position.

9.10.47. The *lower* surface of the leaf was illuminated as before from 7.50 a.m. to 10.12 a.m. Examination then showed four stomata with medium apertures, several with small apertures, and many closed in the field of view. Within 10 minutes of removing the cover-slip the stoma showing the widest opening had closed noticeably. After a further 38 minutes illumination it showed a barely visible opening and all other stomata had closed completely. The 'dummy porometer cup' was left uncovered until the next day.

10.10.47. At 7.55 a.m. the van Tieghem cell was covered as before and the *upper* surface of the leaf illuminated with the 150-W. lamp. At 10.32 a.m. the cover-slip was removed and it was found that many stomata in the field of view had apertures so wide that the ends appeared rounded instead of pointed; many others were of medium aperture. The stomata were noticeably less open 10 minutes after removing the cover-slip, and by 10.50 a.m. many were nearly or quite closed. One stoma maintained a medium opening for another hour.

Expt. 38. *Pelargonium*, Clone 5.

13-15.10.47. This was a repetition of Expt. 37, but whereas in that experiment the 'dummy porometer cup' was covered for the early part of the morning and uncovered later, in the present experiment the 'cup' was left uncovered until about 10.20 a.m. and then covered. The leaf was set up at 10.0 p.m. on 13.10.47; the lower surface of the leaf was illuminated on 14.10.47 and the upper on 15.10.47. The 4-mm. apo. objective was used. No apertures were seen until after the cup had been covered for $1\frac{1}{2}$ hours on 14.10.47, i.e. at about midday, when 24 stomata in the field of view all showed moderate opening. On 15.10.47 no stomatal opening was seen either before the 'cup' was covered or after it had been covered for $1\frac{1}{2}$ hours.

Expt. 39. *Pelargonium*, Clone 5.

15-17.10.47. This was a repetition of Expt. 38, but the cooling screen round the lamp was not used except during the periods when the 'dummy porometer cup' was covered. In this way it was hoped to compensate to some extent for any rise in temperature due to covering the 'cup'. The lower surface of the leaf was illuminated on 16.10.47 and the upper on 17.10.47. The 'cup' was covered from about 10 a.m. to midday on each of the two days. No apertures were seen before the 'cup' had been covered and a few moderately open stomata afterwards.

Expt. 40. Pelargonium, Clone 5.

16.10.47. The plant was placed in a bell jar, partly lined with wet blotting-paper, with the same experimental leaf as in Expt. 38 illuminated by the 150-W. lamp in water screen at a distance of 7 in. The experiment was set up at 9.30 a.m. and at 11.45 a.m. the leaf was cut off and examined with the 8-mm. objective. Most of the stomata appeared closed, but after about 5 minutes a few areas were found where most stomata had moderate apertures. It was thought unlikely that this was due to wilting of the detached leaf, for no further or more general opening was observed later. Such local variations in opening were later indicated by the experiments using the infiltration technique (cf. Fig. 3).

II. Preliminary experiments with the infiltration technique

The experiments described in the preceding section had shown, by direct observation, that under illumination the stomata within a porometer cup in fact opened very much wider than elsewhere on the leaf. They had further established that this effect was in some way due to the enclosure of the portion of leaf and not to the mechanical pressure of the cup on the lower surface or the glass plate on the upper. For the further investigation of the cause of this abnormally wide opening, the infiltration technique as modified by Williams (1949) was used, both because of its rapidity and convenience and because it gave integrated results from a number of stomata far larger than could be observed directly. The infiltration method also enjoys these advantages over Lloyd's method, which in any case would appear from the preceding results to give appreciably wider apertures than those seen by direct observation.

A few details of technique common to all the experiments in this section may be mentioned here.

Where plants or leaves were enclosed under bell jars, the latter were sealed by standing in water and the internal atmosphere was further humidified with strips or a partial lining of wet blotting-paper. The experimental leaf did not touch the wall of the bell jar so that no question of pressure on the leaf was concerned.

For 'glass plate' experiments (see Williams, 1949, p. 317) the leaf was supported on a strip of perspex or ebonite and partly covered with a microscope slide weighted at each end with a 10 or 20 g. weight.

Expt. 41. Pelargonium, Clone 5.

21.10.47. Two potted plants were set up at 7.50 a.m.—one inside a bell jar as in Expt. 40 and the other outside. On each plant the experimental leaf was 5 in. from the filament of the 150-W. lamp. The leaf on the unenclosed plant was partly enclosed between a perspex strip and glass plate. At 10.5 a.m. the bell jar was lifted to place a thermometer with its bulb near the experimental leaf. At 11.25 a.m. this showed 16.3° C., while the temperature outside the bell jar was 15.2° C. Both experimental leaves were then removed and flooded with gentian violet in absolute alcohol. Entry of the dye occurred only in small scattered areas, but these were entirely confined to the leaf from the bell jar and the 'glass plate' area of the other leaf.

Expt. 44. Pelargonium. Bedford College.

22.10.47. This was a repetition of Expt. 41, but with the more responsive Bedford College strain of *Pelargonium*. The experiment ran from 7.50 a.m. to 11.30 a.m., when the temperatures inside and outside the bell jar were 17.7° C. and 16.5° C. respectively. Strips of lower epidermis were taken from each leaf, but not, unfortunately, from the 'glass plate' area, and the leaves were then flooded with alcoholic gentian violet. Practically the whole of the leaf from the bell jar and nearly all the 'glass plate' area of the other leaf became injected. The epidermal strips showed all stomata extremely wide open for the leaf from the bell jar, but medium to small apertures for the other leaf.

Experiments 41 and 44 thus provided further evidence that enclosure and not pressure was the important factor in causing the abnormal opening.

Expts. 42 and 43. Wheat var. 'Charter'.

21.10.47. In these two experiments the 'glass plate' technique was applied to three attached leaves, one in the morning and two in the afternoon, with the adaxial surface illuminated. In the afternoon experiment, injection was confined to the enclosed areas (and the extreme tip of one leaf) and was much heavier when the adaxial surface was flooded than the abaxial. In the morning experiment *both* surfaces were flooded and much heavier injection occurred where the leaf had been enclosed than elsewhere.

This was the first evidence that the wide opening of enclosed stomata occurred in wheat also.

Expt. 45. Wheat var. 'Charter'.

22.10.47. This was a repetition of Expts. 41 and 44, but with *detached* wheat leaves standing with the sheaths in water. The experiment was set up at 12 noon. The leaf outside the bell jar was bent in a helix so that two portions were under the glass plate. The abaxial surfaces of both leaves were illuminated and were 3 in. from the filament of the 150-W. lamp at the nearest points.

At 3.15 p.m. the temperatures were 17.2° C. in the bell jar and 16.9° C. outside. One portion of leaf from under the glass plate was cut off and examined by direct microscopical observation with the 8-mm. objective. Some open stomata were seen in the area from under the glass plate but none outside it. Both halves of the leaf were then flooded with gentian violet and showed only slight injection in the two enclosed areas but none elsewhere except at the extreme tip of the leaf. The leaf from the bell jar showed *no* injection except at the extreme tip.

It was thought that this failure of enclosure within a bell jar to cause wide opening might be connected with the use of a detached leaf and in the next experiment detached leaves of *Pelargonium* were therefore tried.

Expt. 46. Pelargonium. Bedford College.

23.10.47. Three similar small leaves were set up at 8.0 a.m. as follows: *Leaves 1 and 2* were placed in a bell jar and 6 in. from the 150-W. lamp, each with half the lamina darkened by a black paper envelope; *leaf 1* was standing with the petiole in water and *leaf 2* was without water-supply. *Leaf 3* was set up with its petiole in

water and with a 'glass plate', half the area being shaded by black paper placed above the plate and leaf.

At the end of the experiment (11.30 a.m.) the temperatures were 17.2° C. in the bell jar and 16.3° C. outside. *Leaf 3* showed heavy injection in the lighted half of the 'glass plate' area and a little at the edge of the darkened half where the shading may have been inefficient owing to light reflected by the glass. A little scattered injection occurred outside the 'glass plate' area on the illuminated part of the leaf. *Leaf 1* showed only small scattered patches of injection, similar to the last mentioned, on the illuminated half of the lamina. *Leaf 2* was somewhat flaccid and showed heavy injection of most of the illuminated half but none of the darkened half.

Expt. 48. Pelargonium. Bedford College.

24.10.47. A potted plant was set up in a bell jar at 7.40 a.m. as in *Expt. 41*, but with half the experimental leaf shaded by a black paper envelope. At 11.10 a.m., when the experiment was ended, the temperatures were 17.8° C. in the bell jar and 17.0° C. outside. The whole of the illuminated half of the experimental leaf became injected with the gentian violet and practically none of the darkened half.

III. Porometer experiments

The experiments to date had indicated that under illumination the abnormally wide stomatal opening occurred when a portion of leaf was enclosed, though not hermetically sealed, with a *small* volume of air and also when a large number of leaves, as on a potted plant, were enclosed in a bell jar. When, however, only one or two (detached) leaves were enclosed in a bell jar the abnormal opening was not found, except in the case of leaf 2 of *Expt. 46* where wilting might have been the cause. This was considered suggestive of some volatile substance, produced by the leaves, which caused stomatal opening in the light if in sufficient concentration. This might have been an essential oil in the case of *Pelargonium*, but the similar effects found with wheat made this seem improbable. The hypothetical substance was denoted as α .

A series of experiments of the 'glass plate' type was next carried out, both with wheat and *Pelargonium*. In all these experiments small areas under the glass plate were shaded in the attempt to discover if there was any spread of the wide stomatal opening into the darkened areas. The injection did in fact generally spread slightly into these areas, suggesting lateral diffusion of α from the illuminated part of the leaf, but the results were inconclusive, as it was uncertain whether effects of other factors, such as reflected light, had been excluded. They will not, therefore, be presented.

The 'volatile substance' hypothesis was next tested by means of some porometer experiments.

If the hypothesis were correct, by sweeping out a porometer cup with moving air between readings it should be possible to remove any accumulation of α and hence prevent the abnormal stomatal opening or cause closure if it had already occurred. A series of porometer experiments along these lines was therefore begun, using two porometer cups designed and made by the late

H. J. Spencer. As shown in Fig. 1, this design included a side-arm *s* and centre tube *c* by means of which air could be swept close to the enclosed leaf surface. The 'well' surrounding the centre tube could if desired be filled with a desiccating agent, or with wet broken porous tile to humidify the air in the cup. The other modifications of the porometer outlined in the preliminary note (Heath and Williams, 1948) were also used for these experiments, viz. a simple 'gasometer' type of aspirator supplied ordinary laboratory air to the

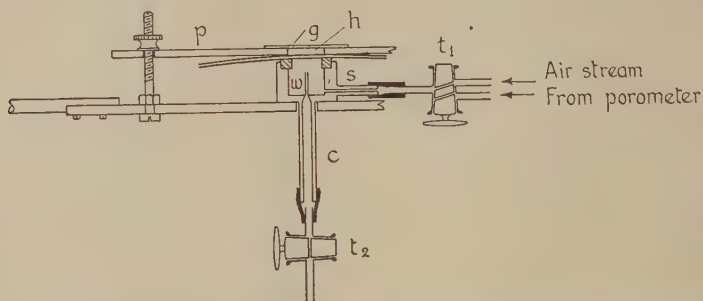


FIG. 1. Porometer cup used for experiments with still or moving air between readings. *s*, side tube connected by 2-way tap, *t*₁, to moving air-stream (dry or moist) or to porometer; *c*, centre tube with tap, *t*₂, which is closed during porometer readings; *w*, well of cup for desiccating agent or wet broken tile; *h*, hole in perspex plate, *p*, to expose upper surface of leaf above cup area; *g*, glass plate to cover *h*.

resistance porometer at a constant *increased* pressure. The aspirator was filled with medicinal paraffin and a special light grade of the same (s.g. 0.83) was used in the two manometers; furthermore, the usual drying tubes (see Heath, 1941, fig. 4, p. 463) were omitted. Thus the porometer could supply to the cup air of the same humidity and composition as that surrounding the rest of the leaf. The pressure gradients were, of course, all reversed as compared with the usual resistance porometer, but the manometer readings were used in the same way, only the signs being changed. The perspex plate pressing the leaf against the porometer cup was drilled with a hole which registered with the area enclosed within the cup (Fig. 1), thus leaving the upper leaf surface above the cup freely exposed to the air. This hole could be covered at will with a small glass plate. Between readings the cups could be swept with laboratory air; usually dry air being used for one cup and wet air for the other. The tubes *s* and *c* of each cup were fitted with taps as shown in Fig. 1. The porometer and the air-supply were both attached to *s* to avoid if possible any mechanical effect of the air-streams impinging directly against the leaf. The moist air-stream was supplied by a 'water blast-pump' worked from the mains and was passed first through a bubbler filled with 'hypo' solution to remove any chlorine and then through a tube filled with wet broken tile. The dry air-stream came from a small electrically driven diaphragm pump and was dried by a calcium chloride tower. A sulphuric acid bubbler was inserted between the pump and tower to indicate the rate of flow. Illumination was supplied by a

150-W. lamp, in running-water screen, 3 in. from the leaf. This gave a light intensity approximating to 1,000 f.c.

Three experiments in this series were carried out, but as the last of them showed all the effects found in the two earlier ones it alone will be described.

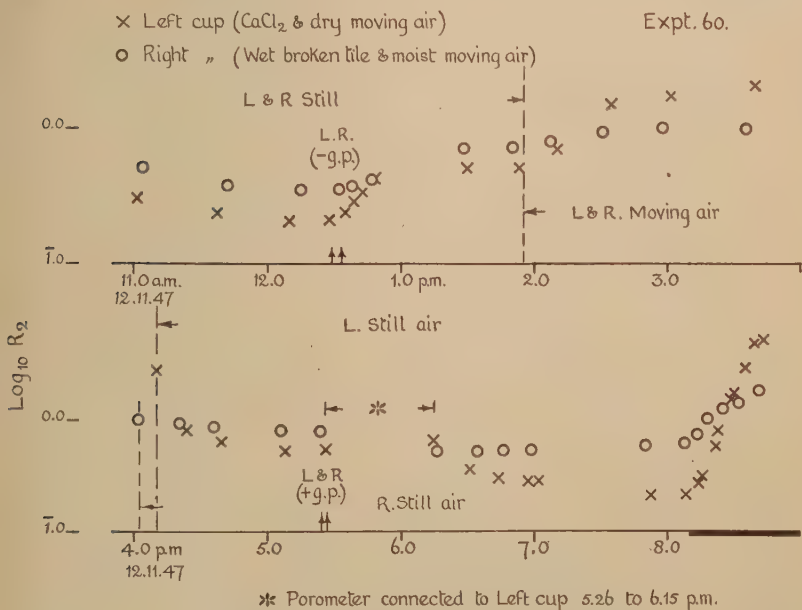


FIG. 2. Expt. 60. 12.11.47. For explanation see text.

Expt. 60. *Pelargonium*. Chelsea.

12.11.47. The two cups were fitted overnight to a leaf 4-in. diameter on a potted plant (not Clone 5). The well of the left cup was filled with calcium chloride and that of the right with wet broken tile. The leaf was left darkened until 9.01 a.m. and then illuminated, the hole over each cup being covered by a glass plate. In Fig. 2, $\log_{10}$ (total resistance) for each cup is plotted against time from 11.00 a.m. when considerable stomatal opening had already occurred. By 12.30 p.m. the resistance for each cup appeared to have reached equilibrium; the importance of attaining equilibrium under one set of conditions before changing to another, in experiments of this type, is stressed in the general discussion. At the times indicated by the arrow heads the glass plates were removed. Stomatal closure followed and was especially rapid in the case of the left (dry) cup. (Clearly there was no question of pressure on the leaf being concerned in this effect.) At 1.55 p.m., when new equilibria had been established, dried laboratory air was blown through the left cup and humidified laboratory air through the right. The air-streams were interrupted for periods of (generally) 2 minutes while the porometer readings were taken. The moving air caused closure in both cups, again more rapid and marked in the 'dry' cup. At about 4 p.m., reverting to 'still air' conditions caused renewed stomatal

opening, especially in the 'dry' cup. At about 5.25 p.m. the glass plates were replaced. The porometer was left connected to the 'dry' cup until 6.15 p.m. in error, and the laboratory air thus blown into the cup and through the leaf was no doubt responsible for the stomatal closure which is shown, for after this was rectified considerable stomatal opening occurred. In the moist cup the fall of resistance as a result of enclosure of the upper surface was much less marked. Finally, at 8.10 p.m. the leaf was darkened. The extremely rapid closure in the 'dry' cup resembles the results obtained by Williams (1950). On removal of the cups the calcium chloride in the left cup was found to be slightly caked and hard but quite dry.

IV. Further experiments with the infiltration technique

A series of further experiments with the infiltration technique, designed to test the 'volatile substance' hypothesis, will now be described. For convenience in describing the various treatments the symbols in Table I are used.

TABLE I

Symbols for treatments in experiments to test 'volatile substance' hypothesis

Enclosed volume	V = large; v = small
Leaf	a = attached; d = detached
Number of enclosed leaves	L = many; l = few or single
Still or moving air (wind)	W = moving; w = still
Humidity	H = high; h = low
Variety (wheat)	Ch = 'Charter'; Ben = 'Bencubbin'
Source (<i>Pelargonium</i>)	C = Chelsea; B = Bedford Coll.

Sets of leaves to be allotted to the different treatments within an experiment were carefully matched in respect of size, colour, and position on the plant. The leaves in each matched set were then allotted at random among the treatments. Replications thus correspond to 'blocks' in a randomized block layout.

Details of technique noted in Section II (p. 35) apply here also.

After Expts. 62 to 68 were completed all the (pressed) leaves within each experiment were graded by inspection in a continuous series for amount of injection, 1 denoting least, and 15 maximum injection. Ties were awarded the means of the appropriate ordinal numbers. For analysis of variance these ranked data have been transformed to normal scores (Fisher and Yates, 1948, Table XX).

Expt. 62. Wheat var. 'Bencubbin'.

13.II.47. Five treatments were set up in triplicate at 1.0 p.m., as follows:

- 1 (Val). A pot of many wheat plants enclosed in a small bell jar. (Three experimental leaves marked.)
- 2 (Val). A pot with two wheat plants, with a total of only three leaves left on, enclosed in a large bell jar.
- 3 (vdL). Three single detached leaves, each in a 9-mm. bore glass tube corked at the top and standing in water.
- 4 (VdL). Three single detached leaves, each standing in water under a separate large bell jar.
- 5 ($Val(a)$). Three detached leaves, standing in water, in the same bell jar as 1 (Val).

Illumination was provided by a 1,000-W. lamp, with reflector, 24 to 36 in. from the leaves. Much water condensed on the walls of all bell jars and tubes, and there is no doubt that in all treatments the air was practically saturated with water vapour.

Between 4.5 and 5.5 p.m. the leaves were removed and flooded with alcoholic gentian violet, one complete replicate at a time. When the bell jar containing treatments 1 and 5 was opened, all the leaves for treatment 1 were cut off and placed in water; the pot was removed and the vessels of detached leaves placed under the bell jar on the bench (unsealed). Thus any accumulated volatile substance could largely have escaped, and at least half an hour elapsed between the testing of the first and third replicate leaves of treatments 1 and 5. The bell jar for treatment 2 was also lifted when the first and the second replicate leaves were tested but was sealed again with water each time.

The grading for amount of injection is shown in Table II.

TABLE II

Experiment 62: 13.II.47. Grading for injection

Replicates	Treatments				
	1 (VaL)	2 (Val)	3 (vdl)	4 (Vdl)	5 (VdL(a))
α	15	5	14	1.5	12
β	10	3	13	8	9
γ	7	5	11	1.5	5

The reduced amounts of injection in the second and third replicates of treatments 1 and 5, as well as the rest of the data, seemed to support the volatile substance hypothesis.

Expt. 63. Wheat vars. 'Charter' and 'Bencubbin'.

15.II.47. There were two treatments (*v* and *V*) which embodied the same technique as treatments 3 and 4 of the last experiment. Each was set up in duplicate, with one leaf of each variety, at 10.45 a.m. The leaves were removed and tested at 1.45 p.m. The grading was: Treatment 3 (*v*), Ch. 4, Ben. 2; Treatment 4 (*V*), Ch. 3, Ben. 1.

For analysis of variance, the data were combined with those from treatments 3 and 4 of Expt. 62. (Treatments 1, 2, and 5 could not be used because of the errors introduced by the delay in testing the second and third replicates.) 'Charter' and 'Bencubbin' were treated as 'blocks' equivalent to α , β , and γ of the former experiment. Treatment 3 (*vdl*) gave a mean normal score of +0.516 and treatment 4 (*Vdl*) of -0.516, the difference being significant at $P < 0.02$.

Expt. 64. Wheat var. 'Bencubbin'.

17.II.47. Three treatments were set up in quadruplicate by 4.15 p.m. Two of these, No. 3 (*vdlw*) and No. 4 (*Vdlw*), were identical with treatments 3 and 4 of Expts. 62 and 63. The third, No. 6 (*vdlW*), in contrast to these, embodied moving air. Four single detached leaves were enclosed in glass tubes standing in water and humid air was blown through each tube continuously at approximately 1.8 l./hr. The arrangement of the moist-air supply was similar to that described in the previous section for the porometer experiments (see p. 38). Condensed moisture in all vessels indicated that the air was practically saturated.

The leaves were removed and flooded with gentian violet after about 4–5 hours, replicate by replicate. The grades of injection and mean normal scores are shown in Table III.

TABLE III

Experiment 64. 17.II.47. Grading for injection

Replicates	Treatments		
	3 (<i>vdlw</i>)	4 (<i>Vdlw</i>)	6 (<i>vdW</i>)
α	11	3.5	6
β	11	3.5	8
γ	11	3.5	3.5
δ	9	1	7
Mean normal score	+1.020	-0.925	-0.095

Significant differences: 3 > 6 and 4 ($P < 0.01$); 6 > 4 ($P < 0.05$)

Treatments 3 and 4 thus fully confirmed the former finding and the results of treatment 6 were also consistent with the hypothesis.

Expt. 65. Wheat var. 'Bencubbin'.

18.II.47. Two treatments, replicated four times, were set up at 1.30 p.m. Treatment 4 (*Vdl*) was as before described; in treatment 7 (*VdL(a)*) the four experimental leaves were standing in water under a small bell jar which also contained a pot of wheat plants and further cut leaves in water. The four leaves from treatment 7 were all removed and tested at 5.20 p.m.; those from treatment 4 up to 5.40 p.m. The results, which were inconclusive, are indicated in Table IV. The reason for the small amount of injection in treatment 7 was not known, but the potted plants were old and in poor condition.

TABLE IV

Experiment 65. 18.II.47. Grading for injection

Replicates	Treatments	
	4 (<i>Vdl</i>)	7 (<i>VdL(a)</i>)
α	7	6
β	1.5	4.5
γ	3	8
δ	1.5	4.5
Mean normal score	-0.473	+0.473

Difference not significant ($P > 0.1$).

Expt. 66. Wheat var. 'Bencubbin'.

19.II.47. Four treatments in triplicate were set up by 12.50 p.m. Twelve glass tubes, 38 cm. long and 1.5 cm. bore, were arranged horizontally on a rack. At one end of each an attached 'elbow' of glass tubing enclosed the sheath of the experimental leaf which dipped in a tray of water; the other end was either stoppered or an air-supply was attached. Illumination was provided by a 1,000-W. lamp and reflector at 32–35 in. distance. The treatments were as follows:

- 3 (*vwH*). Essentially the same as treatment 3 of previous experiments—high humidity and still air.

6 (*vWH*). Essentially similar to treatment 6 of Expt. 64—high humidity, moving air.

8 (*vwh*). Low humidity and still air. The leaf lay on a thick layer of dry silica gel.

9 (*vWh*). Low humidity and moving air. A stream of dry air was blown through the tube (for air-supply details see p. 38).

The moving air to each tube in treatments 6 and 9 was supplied at approximately 2.7 l./hr. up to 3.50 p.m. and thereafter at 4.2 l./hr. Much condensed moisture collected in the tubes of treatments 3 and 6, and some also below the leaves in treatment 9 β and γ .

Between 6.10 p.m. and 6.50 p.m. the leaves were tested. All three leaves of treatment 8 were badly wilted and the result of this treatment must therefore be discounted. The graded results are shown in Table V.

TABLE V

Experiment 66. 19.II.47. Grading for injection

Replicates	Treatments			
	3 (<i>vwH</i>)	6 (<i>vWH</i>)	8 (<i>vwh</i>)	9 (<i>vWh</i>)
α	12	4	1.5	7
β	11	5	1.5	8.5
γ	10	8.5	3	6
Mean normal score	+1.180	-0.142	-1.180	+0.142

Significant differences: 3 > 6 and 9 ($P < 0.01$); 6 and 9 > 8 ($P < 0.01$).

Expt. 68. Wheat vars. 'Bencubbin' and 'Charter'.

20.II.47. The treatments were the same as treatments 3 and 8 of Expt. 66, except that a strip of black paper was inserted under each leaf and in the case of treatment 8 between the leaf and silica gel. There were five replicates—two of var. 'Bencubbin' and three of var. 'Charter'. The experiment was set up by 3.30 p.m. and the leaves were tested between 8.5 and 8.55 p.m. The results are shown in Table VI. As between varieties the leaves were not matched and the varietal comparison is not valid therefore.

TABLE VI

Experiment 68. 20.II.47. Grading for injection

Replicates	Treatments	
	3 (<i>vwH</i>)	8 (<i>vwh</i>)
α Ch.	7.5	3
γ Ch.	6	1.5
ϵ Ch.	10	7.5
β Ben.	5	1.5
δ Ben.	9	4
Mean normal score	+0.612	-0.612

Difference significant at $P < 0.001$.

Expt. 67. Pelargonium. Chelsea and Bedford College.

20.II.47. This experiment was essentially a repetition of Expt. 65 but with *Pelargonium*. Treatment 4 (*Vdl*) was as described for Expt. 62; in treatment 7 (*Vdl(a)*) the four experimental leaves, in test-tubes of water, were supported in

front of three potted plants and all enclosed under a bell jar. Two of the four leaves were from plants of the Bedford College strain and two from Chelsea Physic Garden (not Clone 5). Some mutual shading of the experimental leaves occurred. The experiment was set up between 8.25 and 8.50 a.m. and was terminated at 1.33 p.m. All the four leaves of treatment 7 were tested as quickly as possible after lifting the bell jar. The results are tabulated in Table VII and indicated in Fig. 3.

TABLE VII
Experiment 67. 20.11.47. Grading for injection

Replicates	Treatments	
	4 (<i>Vdl</i>)	7 (<i>VdL(a)</i>)
C { α	1	3.5
{ β	2	3.5
B { α	5.5	8
{ β	5.5	7
Mean normal scores	-0.413	+0.413
Difference significant at $P < 0.02$.		

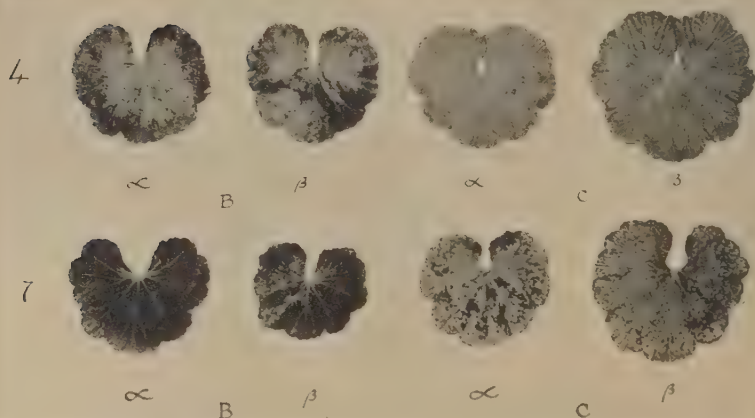


FIG. 3. *Pelargonium* leaves from Expt. 67 after infiltration with alcoholic gentian violet (leaves dried and pressed before photographing). 1. Treatment 4 (*Vdl*) (see text). Single detached leaves enclosed under bell jars and illuminated for about 5 hours. 2. Treatment 7 (*VdL(a)*) (see text). Detached leaves similarly enclosed and illuminated but together with potted plants. B. Bedford College strain ('Paired leaves'). C. Chelsea Physic Garden strain (not Clone 5) ('Paired leaves'). In each pair injection is much heavier in the leaf from the crowded bell jar.

V. Preliminary discussion

In reviewing the work so far it is desirable to list the respects in which conditions within a porometer cup under illumination differ from those outside and consider which of these might be held responsible for the abnormal stomatal opening.

1. The portion of leaf enclosed in the cup is (usually) illuminated only by

light transmitted from above, since the cup, if of opaque material, shades the lower surface from all direct light. This is a very old criticism of the porometer and was refuted by Knight (1922); leaves normally receive most of their light on the upper surface. In any case it seems very improbable that a *reduction* of illumination of the lower surface would cause *wider* stomatal opening within the cup, for only moderate intensities were used in these experiments.

2. The temperature within the cup may be higher than that outside. However, similar abnormal opening was obtained in the bell-jar experiments with differences of temperature not exceeding 1.5° C. The differences in aperture can therefore occur in the absence of appreciable temperature differences.

3. The upper surface of the leaf above the cup is (usually) touching a glass plate and the cup itself presses the leaf against this plate round the perimeter of the enclosed area. The experiments with 'dummy porometer cups' in Section I and the various experiments with bell jars showed clearly, however, that enclosure in a restricted volume, and not pressure against a glass plate, was the important factor in causing abnormal opening. The effects of removing the glass plates in the porometer experiments (e.g. Expt. 60) provided some supporting evidence for this.

4. The portion of leaf enclosed in the cup is in still air if the porometer is disconnected between readings, whereas the rest of the leaf is exposed to the ubiquitous draughts and air movements of a laboratory. Bell-jar experiments, such as Expt. 67, with many and few leaves similarly enclosed showed that air movement *per se* was not the important factor.

5. Enclosure of a portion of the leaf in the restricted volume of the cup may result in a number of changes in the composition of the enclosed air. These changes will in most cases be accelerated if, as with the normal type of porometer, air is drawn through the intercellular space system into the cup:

a. The air in the cup will rapidly become saturated with water vapour. The experiments described in Section IV showed, however, that the differences of stomatal aperture could occur where humid air was used throughout. Furthermore, in porometer experiments (e.g. Expt. 60) with still air between readings, similar low resistances were obtained under both moist and dry conditions, and Williams (1950) has shown by Lloyd's method that in such experiments the abnormal opening occurred in both the 'wet' and 'dry' cups.

b. The oxygen concentration in the cup will rise owing to photosynthesis. This seemed unlikely to be important, as 20 per cent. oxygen is normally present and the increase could not be large.

c. The carbon dioxide concentration will fall for the same reason. This also seemed most unlikely to be of importance, for the available range was only from the normal 0.03 per cent. to zero.

d. The concentration of ethereal oils (in the case of *Pelargonium*) and any other volatile substances given off by the leaf will rise. In view of the essentially similar effects found in *Pelargonium* and wheat it seemed unlikely that the ethereal oils of the former constituted the important factor.

All the experimental results so far obtained were consistent with the

'volatile substance' hypothesis put forward on p. 37, though in the case of wheat there was some evidence of an additional closing effect of low humidity (p. 43) and in *Pelargonium* the responses appeared more rapid under dry conditions (p. 39). Whether α was effective only in the light, or was produced only in the light but effective also in darkness, could not be decided on the evidence available, as the experiments mentioned on p. 37 which would have tested this were not conclusive. In the porometer experiments, not only the effect of moving air, whether wet or dry, seemed to support the hypothesis, but also the effects of removing and replacing the glass plates. The latter presumably affected the total resistance by causing closure or opening of the upper stomata. The statistically significant results presented in Section IV may be summarized thus:

- (1) Heavier injection with many leaves (L) than with few or single leaves (l) enclosed in a similar large volume of humid still air (Expt. 67, p. 43; supporting evidence from Expt. 62, p. 40, and Expt. 65, p. 42).
- (2) Heavier injection with single leaves in a small (v) than in a large (V) volume of humid still air (Expts. 62 and 63, pp. 40 and 41, Expt. 64, p. 41).
- (3) Heavier injection with single leaves in a small volume of humid still air (w) than in humid moving air (W) (Expt. 64, p. 41; Expt. 66, p. 42).
- (4) Heavier injection with single leaves in a small volume of humid still air (H) than of dry still air (h) (Expt. 68, p. 43).

All these results, except the last-mentioned humidity effect, seemed to support the hypothesis; as did also Darwin's (1898) finding that removing plants from a presumably crowded greenhouse to the laboratory caused stomatal closure, but that enclosure in bell jars caused the stomata to remain open or reopen. Darwin himself attributed these results to humidity differences.

None of the data, however, conflicted with two other hypotheses indicated above, both of which seemed very improbable, viz. that the abnormal opening was brought about either by the increase of oxygen concentration or the decrease of carbon dioxide concentration. Since a simple but crucial experiment was available to test the latter hypothesis it was decided to carry this out before embarking upon the collection and identification of α . This experiment is described in the next section.

VI. Further porometer experiments to test the carbon dioxide hypothesis

Details of technique were as described in Section III except that flow-meters were used to measure the rates of flow of the moist and dry air-streams; for the latter a phosphorus pentoxide and a silica-gel tower were used in series. For providing carbon dioxide-free air a 'Sofnolite' tower was by-passed into the dry air-stream immediately before the porometer cup and a spiral bubbler containing normal caustic soda solution was similarly inserted in the line for the moist-air stream. After Expt. 69 a small 'Sofnolite' tube was by-passed into the line from the porometer to the cup during periods of carbon

dioxide-free air-flow. Thus no carbon dioxide was supplied to the cup from the porometer when taking readings during such periods.

For the wheat experiments carried out by Dr. F. L. Milthorpe a somewhat similar technique was used, but with a special rectangular cup similar to one which will be described in detail in the next paper of this series. This cup enabled both upper and lower leaf surfaces to be swept with air of controlled composition. The light intensity was very much lower than in the *Pelargonium* experiments and approximated to 200 f.c.

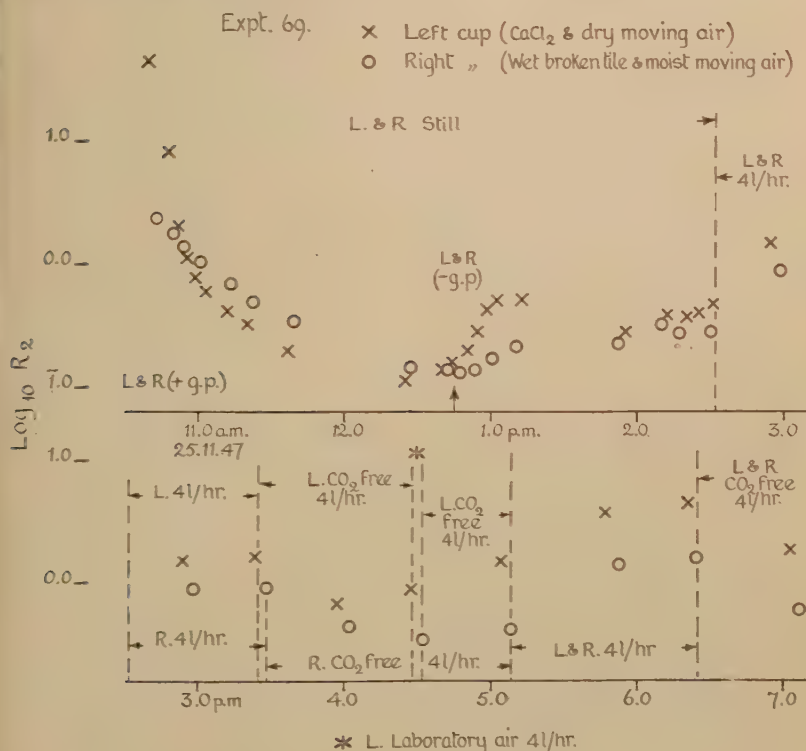


FIG. 4. Expt. 69. 25.11.47. For explanation see text.

Expt. 69. *Pelargonium*. Bedford College.

25.11.47. The two cups were fitted overnight as described for Expt. 60 and the leaf darkened. In Fig. 4, stomatal opening under illumination in still air, with the holes above the cups covered by glass plates, can be seen to have been especially rapid in the 'dry' left cup. At 12.45 p.m. both glass plates were removed and the very marked closure in the 'dry' cup was associated with draughts caused by the opening of a window. At 2.32 p.m. the air-streams were started at 4 l. hr. with dry laboratory air through the left cup and moist through the right. Closure to new

approximate equilibria resulted. At about 3.25 p.m. carbon dioxide-free air was substituted for ordinary laboratory air and marked stomatal *opening* resulted in both cups. Later there was a tendency for the usual afternoon closure to occur in both cups and this was probably accentuated in the 'dry' cup by 4 minutes' flow of ordinary air given in error. From 5.08 p.m. both cups were swept with ordinary air and further stomatal closure resulted. Changing back to carbon dioxide-free air at 6.25 p.m. resulted in renewed opening, in spite of the late hour. The experiment was continued with illumination until 9.13 p.m. but a comparison of still air and carbon dioxide-free air gave small and not very consistent differences and the data are not presented. On darkening the leaf, rapid closure occurred, especially in the 'dry' cup.

Expt. 71. Pelargonium. Bedford College.

27.11.47. The two cups, with empty 'wells', were fitted at 11.30 p.m. on 26.11.47 to a leaf 3 in. in diameter. Thus with still air the conditions were the same in both cups. The leaf was illuminated, from 8.57 a.m., with glass plates covering the holes above the cups, and still air. By midday the resistance for each cup had fallen to a nearly steady value (Fig. 5) and from 12.35 p.m. carbon dioxide-free air was passed through at 4 l./hr.—dry through the left cup and moist through the right. There was some indication that this may have caused slight further stomatal opening in the left cup; certainly no closure resulted. Substituting ordinary laboratory air for carbon dioxide-free air (1.11 p.m. and 1.15 p.m.) caused marked closure which was especially rapid in the dry air. Removal of the glass plates at about 3.25 p.m. caused a further increase in the resistance for the left cup; as the right had not reached an equilibrium value the increased resistance it showed may or may not have been due to exposure of the upper surface and consequent closure of the stomata there. There followed periods with ordinary air supplied at various rates of flow. These gave little indication that rate of flow affected the stomata (Fig. 5). At about 5 p.m. carbon dioxide-free air was substituted for ordinary air with a rate of flow of 1 l./hr. By about 6 p.m. (not shown in Fig. 5) the resistances for both cups had fallen markedly, especially the left, and at about 6.24 p.m. (the next points plotted) slight stomatal closure had followed.

Increasing the rate of flow of carbon dioxide-free air to 4 l./hr. was followed by slight changes of resistance in opposite directions for the two cups. Further periods of ordinary and carbon dioxide-free air, the former both at 4 l./hr. and 1 l./hr., confirmed the previous findings in all respects. On darkening the leaf (9.58 p.m.) partial closure occurred and on stopping the flow of carbon dioxide-free air marked further closure resulted. The leaf was left darkened overnight with the holes above the cups covered.

It was concluded from this experiment that a flow of 1 l./hr. swept the cup efficiently and in later work 4 l./hr. was adopted as standard.

28.11.47. Readings were begun at 8.54 a.m. with the leaf in darkness and still air in the cups. On changing to moving ordinary air, marked closure resulted with the dry air. The reason for this is not clear (see general discussion, p. 58). At 10.21 a.m. changing to carbon dioxide-free air caused stomatal opening and at about 11.10 a.m. the converse change caused renewed closure, in both cases most rapid with the dry air. At 12.8 p.m. the leaf was illuminated and the usual stomatal opening followed.

It seemed, therefore, that the effects of carbon dioxide-free and ordinary

moving air, both dry and moist, were essentially the same in darkness as in light. In the next porometer experiment further evidence on this question was collected.

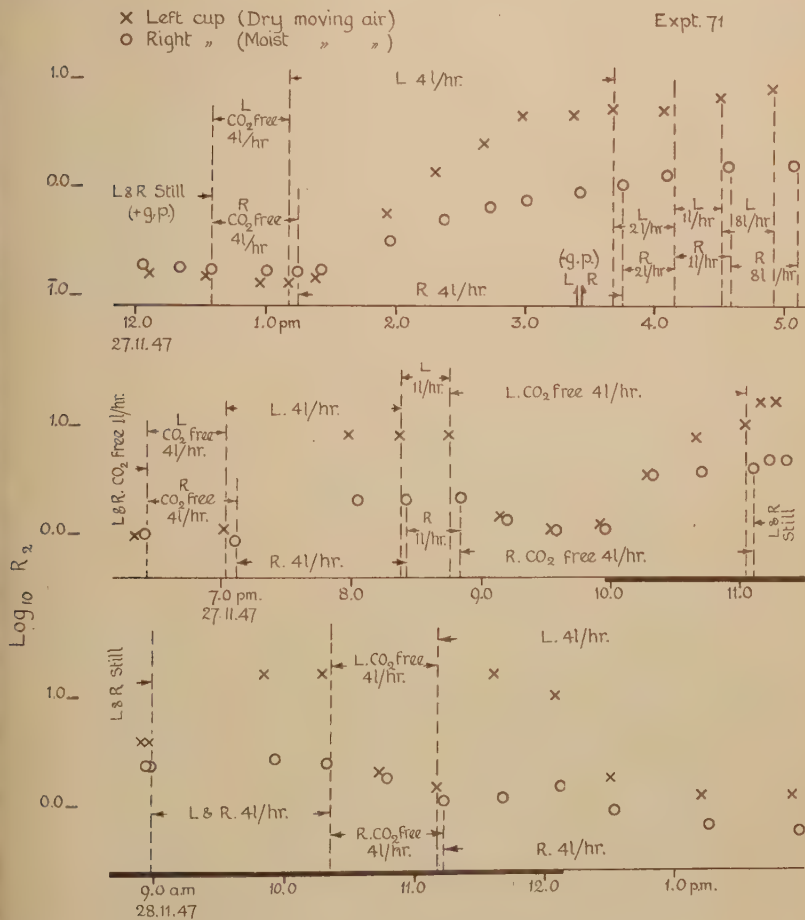


FIG. 5. Expt. 71. 27–28.11.47. For explanation see text.

Expt. 73. *Pelargonium*. Clone 5.

2.12.47. The two cups were fitted as in Expt. 71, at about midnight, on a leaf $3\frac{3}{4}$ in. in diameter, which was then left darkened with the holes in the perspex strips covered. In this experiment the holes were not covered again after 11.48 a.m., 2.12.47, either during periods of illumination or darkness. Dry moving air was used for both cups, which thus provided duplicate data. Readings were begun at 8.38 a.m. with still air, and moving ordinary air was then swept through the cups (Fig. 6). This caused an even greater increase in resistance than that under comparable conditions in Expt. 71 (Fig. 5, left cup, 8.54–10.17 a.m., 28.11.47). The readings of the

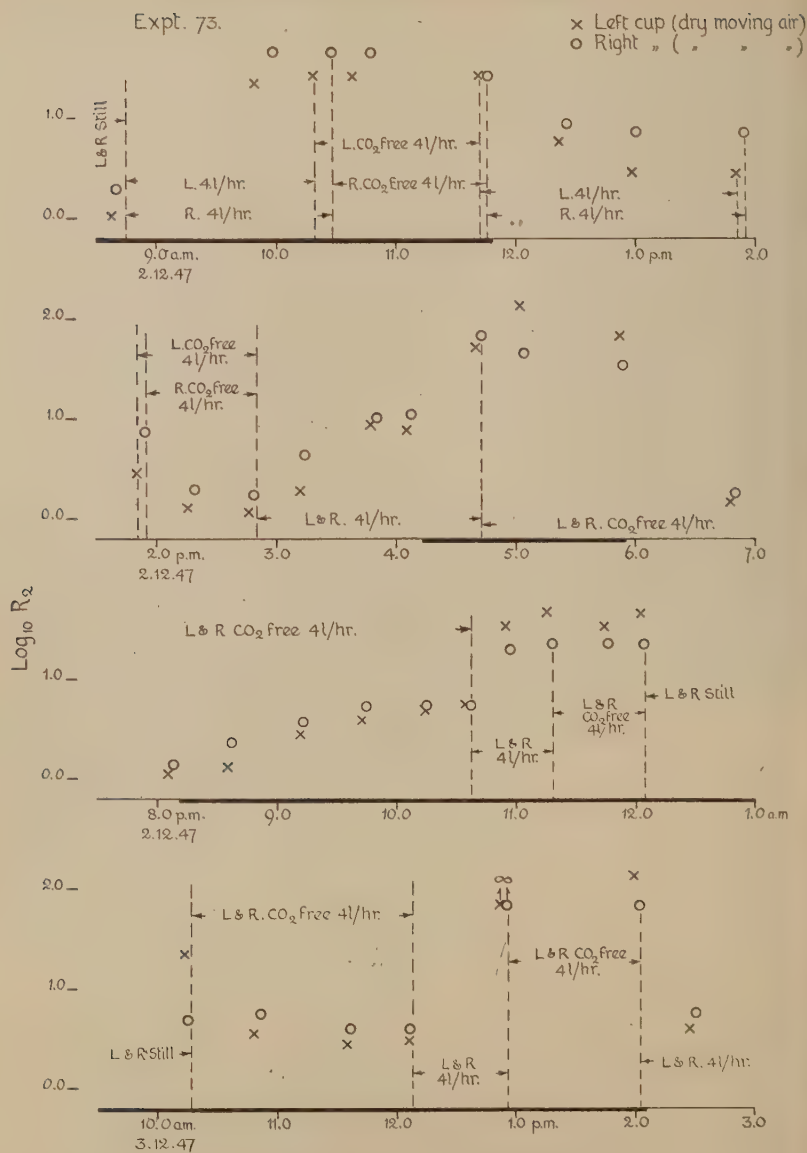


FIG. 6. Expt. 73. 2-3.12.47. For explanation see text.

two manometers of the resistance porometer, in fact, now only differed by approximately 0.5 mm. or about the limit of accuracy of reading, so that there was little evidence that the resistance differed from infinity and the stomata must have been

practically closed. This is a most unusual result to obtain with *Pelargonium*, but in the event proved very fortunate. At about 10.25 a.m. carbon dioxide-free air was substituted for ordinary air, but no significant fall in resistance followed. This result thus differed radically from that in Expt. 71, where marked stomatal opening was caused (Fig. 5, 10.11–11.13 a.m., 28.11.47). Illumination from 11.48 a.m. caused normal stomatal opening, with ordinary air passing through the cups; changing to carbon dioxide-free air and back to normal air subsequently caused the changes of resistance which were now expected. Darkening the leaf at 4.12 p.m. with ordinary air passing through the cups again caused practically complete stomatal closure, and carbon dioxide-free air from 4.43 p.m. again failed to produce any significant change; under illumination from 5.55 p.m. the stomata once more opened normally.

From 8.11 p.m. the experiment was carried out in the reverse order, and the leaf was then darkened with carbon dioxide-free air passing through the cups. Only partial stomatal closure resulted, as in Expt. 71 (Fig. 5, 9.57–11.2 p.m., 27.11.47). Changing to normal air at 10.38 p.m. then caused practically complete closure and, subsequently, carbon dioxide-free air from 11.18 p.m. failed to cause opening. The leaf was left darkened overnight and this sequence repeated in the morning.

3.12.47. The resistances in still air at about 10.15 a.m. were appreciably lower than after the treatment with ordinary air the previous night, and carbon dioxide-free air from 10.17 a.m. caused pronounced stomatal opening. Ordinary air from 12.8 p.m. apparently closed the stomata completely ($R_2 = \infty$), and carbon dioxide-free air from 12.56 p.m. then failed to reopen them. Illumination from 2.6 p.m. gave the usual stomatal opening.

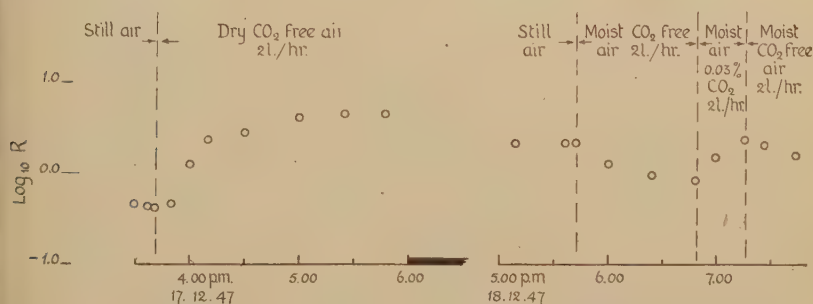


FIG. 7. Expt. of 17–18.12.47. For explanation see text.

Expt. of 17–18.12.47. Wheat var. 'Charter'.

This was one of several preliminary porometer experiments carried out by Dr. F. L. Milthorpe to discover whether the same effects were found with the wheat leaf. A portion of the record for 17.12.47 is shown in Fig. 7. It is seen that the change from still air to dry carbon dioxide-free air at 2 l./hr. caused rapid and pronounced stomatal closure. This was very suggestive of a direct closing effect of dry air. The leaf was left in darkness overnight and the stomata were then allowed to open in still air in the light. The change from still air to moist carbon dioxide-free air was then made (Fig. 7), and, unlike the result with dry air noted above, very marked

opening was caused. Substituting ordinary moist air at the same rate of flow caused even more rapid stomatal closure, and carbon dioxide-free air then caused renewed opening. These effects were also found in other experiments with the same treatments.

VII. Further experiments with the infiltration technique to test the carbon dioxide hypothesis

Six confirmatory experiments of a simple but rather striking kind, which is now in use as a class experiment, were carried out—three with *Pelargonium* and three (by Dr. F. L. Milthorpe) with wheat. 'Paired leaves' were used and allotted at random to the two treatments, which in each experiment were replicated three times. Each leaf was supported in a test-tube of water under a separate large bell jar, sealed and humidified as in Sections II and IV, and was illuminated by the 1,000-W. lamp with reflector at 31–33-in. distance. The treatment difference lay in half of the bell jars each enclosing a large (8-in. diam.) dish containing about 300 c.c. of 2N soda solution. The beaker supporting the leaf stood in this dish so that the lamina was over the solution. After 2–5 hours' illumination the leaves were removed and flooded with alcoholic gentian violet, pair by pair.

Expts. 70, 72, and 74. *Pelargonium*. Chelsea, Clone 5, and Bedford College.

26 and 28.11.47 and 4.12.47. The eighteen leaves from these three experiments were graded in respect of amount of injection and the significance tested as described on p. 40. The ranked data are presented in Table VIII together with details as to the strains used.

TABLE VIII

Experiments 70, 72, and 74. 26 and 28.11.47 and 4.12.47. Grading for injection

Expt. No.	Duration (Hours)	Replicate	<i>Pelargonium</i> strain	Treatment	
				1(+CO ₂)	2(-CO ₂)
70	4	α	Chelsea (not Clone 5)	6	14
	"	β	Bedford College	7	17
	"	γ	Chelsea (not Clone 5)	2.5	12.5
72	4	α	Bedford College	17	17
	"	β	"	15	12.5
	"	γ	Chelsea, Clone 5	5	8.5
74	2	α	"	4	11
	"	β	"	2.5	8.5
	"	γ	Bedford College	1	10
Mean normal score				-0.543	+0.543

Difference significant at $P < 0.01$.

In Expt. 72 both the α leaves were very heavily injected and the experiment was clearly allowed to run rather too long for the Bedford College strain; the β pair showed a difference in the opposite direction from most and this was solely due to less injection of the -CO₂ leaf down one side which had been bent away from the

light. For Expt. 74 the duration was reduced to 2 hours and this gave most striking and consistent differences (Fig. 8).

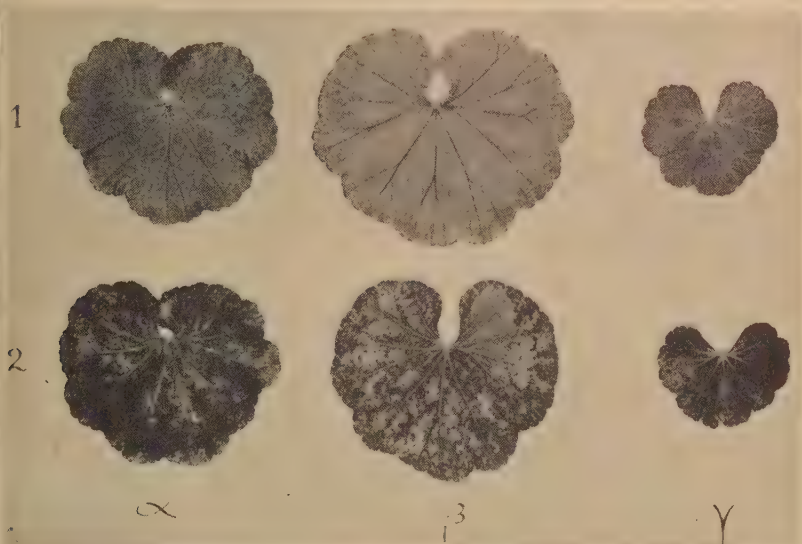


FIG. 8. *Pelargonium* leaves from Expt. 74 after infiltration with alcoholic gentian violet (leaves dried and pressed before photographing). 1. $+CO_2$. Single detached leaves enclosed under bell jars and illuminated for about 2 hours. 2. $-CO_2$. Single detached leaves similarly enclosed and illuminated but in the presence of 2N soda solution. α and β . 'Clone 5' ('Paired leaves'). γ . Bedford College strain ('Paired leaves'). In each pair, injection is much heavier in the $-CO_2$ leaf.

Expts. 75, 76, and 77. Wheat vars. 'Charter' and 'Bencubbin'.

5 and 6.12.47. The wheat leaves were maintained in an erect position by means of a glass rod and rubber band. The eighteen leaves were graded as in the *Pelargonium* experiments and the data are similarly presented in Table IX. The significance was tested as before described.

TABLE IX

Experiments 75, 76, and 77. 5 and 6.12.47. Grading for injection

Expt. No.	Duration (Hours)	Replicate	Wheat variety	Treatment	
				1 ($+CO_2$)	2 ($-CO_2$)
75	3	α	Charter	1	12.5
	"	β	"	5	9
	"	γ	Bencubbin	5	11
76	2	α	Charter	12.5	2.5
	"	β	"	10	14.5
	"	γ	"	8	14.5
77	5	α	"	2.5	17
	"	β	"	5	17
	"	γ	"	7	17
Mean normal score				-0.639	+0.639

Difference significant at $P < 0.05$.

The results of Expt. 75 are illustrated in Fig. 9.



FIG. 9. Wheat leaves from Expt. 75 after infiltration. 1. $+CO_2$. 2. $-CO_2$. Treatments as in Fig. 8, but illumination for about 3 hours. α and β , var. 'Charter' ('Paired leaves'). γ var. 'Bencubbin' ('Paired leaves'). In each pair, injection is heavier in the $-CO_2$ leaf.

GENERAL DISCUSSION

1. *The carbon dioxide effect*

The results presented in Sections VI and VII, supported by the other data in this paper, would seem to leave virtually no doubt that the abnormally wide opening of stomata within a porometer cup is caused by reduction of the carbon dioxide content of the enclosed air brought about by photosynthesis. Not only do the porometer experiments with moving carbon dioxide-free and ordinary air disprove the 'volatile substance' hypothesis but also the 'oxygen' hypothesis, for any accumulation of either α or oxygen should have been swept away as efficiently by carbon dioxide-free air as by ordinary air. In the bell-jar experiments of Section VII, likewise, at least as much of either α or

oxygen should have accumulated in the bell jars without caustic soda as in those with it; unless, of course, a much greater solubility in water than in caustic soda solution were involved, and even so in both treatments there was a considerable free water surface exposed. Apart from a carbon dioxide effect the only remaining possibility seems to be that some other substance in the air-supply caused closure. Such a substance would have to be absorbed by caustic soda or soda-lime, but not by the silica gel, phosphorus pentoxide and calcium chloride used to dry the air, nor by the sodium thiosulphate used to remove chlorine from the moist air-stream. Furthermore, it would have to be destroyed or removed by enclosure in a small volume with an illuminated leaf. Such a substance must, moreover, be of wide occurrence, for the partial closure of stomata freely exposed to the air was found at Bedford College (Williams, 1949) and in two laboratories at the Imperial College. The gas supply in the latter was cut off and there was no tobacco smoke. It must be admitted that on the present evidence the carbon dioxide hypothesis seems by far the most plausible. The volatile substance x has, in fact, proved to be like 'phlogiston', a negative quantity—minus-carbon dioxide instead of minus-oxygen.

The results of Expt. 73 indicate that, at least in *Pelargonium*, it is the carbon dioxide concentration in the substomatal cavity and not at the outer surface of the guard cells that controls their movement, for with the stomata practically shut external concentration is without effect. These findings again raise the question of whether the light response of the stomata may not be entirely due to the removal of carbon dioxide from the intercellular spaces by photosynthesis and the dark response to its accumulation inside the leaf owing to respiration, as has been suggested by Stahl (1919), Sayre (1926), Scarth (1932), and others. It is now clear, however, that in addition to the carbon dioxide of respiration the external supply plays a large part in controlling stomatal movement, especially in the light, and the importance of wind becomes manifest.

The method by which such small changes of carbon dioxide concentration, within the range 0.03 per cent. to zero, control stomatal movement is not at all clear and much further investigation is needed. It has been shown experimentally (Heath, 1938) that the opening force in the stoma is supplied ultimately by the turgor of the guard cells. The intermediate steps between the carbon dioxide concentration in the substomatal cavity and the turgor changes which cause the movements of the guard cells are quite uncertain. The evidence for the usual starch $\rightleftharpoons$ sugar hypothesis, whether controlled through pH change or in any other way, is not convincing as far as the light-dark response is concerned (Heath, 1949). Nor, indeed, does it seem probable that such small changes of carbon dioxide concentration could appreciably affect the pH in the guard cells, which must contain buffer systems. Here, however, the possibility of organic acid formation, which has been found by Bonner and Bonner (1948) to increase greatly with carbon dioxide concentration over a similar range in leaves of *Bryophyllum* in darkness at low temperature, must not be overlooked.

2. *Relevance to other investigations with the porometer*

The discoveries of the abnormally wide opening of stomata when in a confined space and of their extreme sensitivity to changes of carbon dioxide concentration below the normal 0.03 per cent. have been found to furnish plausible explanations of a number of hitherto obscure phenomena observed during some 30 years of stomatal research at this college. In particular may be cited the results of Knight (1917) for the relations between transpiration, water content, and stomatal movement under conditions of still air and wind, which are discussed by Gregory *et al.* (1950a) in the light of the new findings. Another example is provided by the so-called 'Knight effect', or apparent shock closure due to fitting a porometer cup, which has been discussed at length by Williams (1949). It is clear that if this response to carbon dioxide is of at all general occurrence, and in addition to the two very remote genera dealt with in this paper there are indications of similar effects in *Eucharis*, *Ficus*, and *Begonia* (Williams, loc. cit.), the gravest doubt attaches to the results of experiments in which stomatal behaviour within a permanently attached and unswept porometer cup is compared with the transpiration, assimilation, or water content of the leaf as a whole. The experiments of Knight (1917) already mentioned are examples of this. As far as assimilation experiments are concerned, even the penetrating researches of Maskell (1928) would appear open to this criticism, for he apparently left his porometer cup in position throughout an experiment, although he detached it and replaced it in 'surveying' the leaf at the beginning and end (loc. cit., p. 490). His consistent results suggest, however, either that cherry laurel stomata do not show these effects or else that the stomata outside the cup must have followed a diurnal rhythm similar to that found inside it, even though the magnitudes of the apertures may have differed. In the author's 'over' assimilation experiments (Heath, 1939, 1949a) the assimilation chamber was itself the porometer cup so that the same stomata were concerned in both measurements; furthermore, by means of other chambers swept with air of the same known carbon dioxide content, identical conditions were maintained between porometer readings within, above, and surrounding the 'cup'. At least as an approximation, therefore, the type of analysis developed by Penman (1942) should be applicable to the porometer data. The full importance of these precautions was, of course, not realized at the time the experiments were carried out, although the advantage claimed for the use of the assimilation chamber as porometer cup (originally suggested by Prof. F. G. Gregory) was that 'The question of whether stomatal movement is synchronous in different parts of the leaf does not then arise' (Heath, 1939).

The maintenance of the same conditions within, above, and outside the cup area is likely to be of importance even in semi-quantitative use of the porometer as in the investigation of the dynamics of stomatal movement where questions of rate of change or the shape of the curve are concerned. In amphistomatous leaves with relatively few upper stomata, such as *Pelargonium*, and even more with hypostomatous leaves, relative closure of the lower stomata exterior to the

cup area will have large effects upon the porometer readings, especially at the larger apertures; moreover, in amphistomatous leaves, as the stomata open, those above the cup have an increasingly important effect upon the total resistance measured (Heath, 1941). Examples of this last effect can be seen in the porometer experiments, where the removal or replacement of the glass plates over the holes in the perspex strips presumably caused closure or opening of the upper stomata; these results emphasize the importance of having the leaf surface above the cup exposed when a detachable cup is used, or swept with air of the same composition as that passed through a 'swept' cup. It seems almost certain that stomata enclosed in an unswept cup and those exposed on the rest of the leaf will both open and close at different rates. Thus, on darkening the leaf after prolonged illumination, the enclosed stomata might be expected at first to close more slowly than the exterior stomata, until the carbon dioxide in the cup had built up to the normal 0.03 per cent., and then more rapidly as higher concentrations accumulated. After prolonged darkness the stomata within the cup are likely to be wider open than those outside (p. 58); after a somewhat shorter exposure to darkness the converse situation seems likely to occur. In either case the carbon dioxide concentration in the cup will probably be above the normal and on illumination the rates of opening be differentially affected. As the carbon dioxide in the cup falls below the normal the rate of opening within the cup seems likely to exceed that outside and it will certainly proceed to a wider mean aperture at equilibrium. Furthermore, if the stomata above the cup area are enclosed under a glass plate in contact with the leaf, the changes of carbon dioxide concentration both in light and in darkness are likely to occur more rapidly there than in the cup owing to the much smaller volume concerned (although the lack of a sealing washer and the smaller number of stomata will tend to compensate for this). These upper stomata are likely, therefore, to follow yet another pair of opening and closing curves. In addition to these suggested carbon dioxide effects, further complications are imposed by the less rapid responses of stomata under conditions of high humidity recorded by Williams (1950) and in the present paper. Whatever the integrated effects of these factors may be, it seems almost certain that over much of the range of resistance measured the same porometer readings obtained during opening in the light and closure in the darkness respectively will correspond to different apertures within the cup. The opening and closing curves found will thus probably be distorted in different ways and any conclusions based upon their shapes or slopes may be fallacious.

It would seem that all quantitative and semi-quantitative porometer investigations already carried out with a permanently attached unswept cup require checking with a detachable or swept cup and the upper surface above the cup exposed; this includes the author's determinations of 'mesophyll resistance' for *Pelargonium* and *Begonia*, and the comparison of the 'leaf chamber' and small cup (Heath, 1941). Results obtained with the porometer in its traditional form are, however, valid for the enclosed stomata so far as purely qualitative

indication of opening or closure in response to light or darkness is concerned (though *not* for the response to wind, humidity, or other factors operative outside the cup only). The stomata within the cup do respond to light, apparently to a far greater extent than those outside.

3. *Subsidiary observations*

The response to carbon dioxide reported here clearly demands a much more rigorous and quantitative investigation, and the preliminary experiments of such an investigation will be described in the next paper of this series; until it has made considerable progress the interpretation of some of the present results must remain in doubt. In particular may be mentioned the very marked stomatal opening found in wheat on changing from still air to moist carbon dioxide-free air (p. 51). This may perhaps have been associated with a low assimilation rate under the low light intensity used (200 f.c.). Slight indications of a similar effect with *Pelargonium*, but with *dry* carbon dioxide-free air, were found in Expt. 71 and another experiment not presented. Another curious result is the marked closure caused in *Pelargonium* by the change in darkness from still air to moving dry ordinary air (Expts. 71 and 73). The carbon dioxide content in the cup in still air would be expected to exceed greatly the normal 0.03 per cent. of the moving air; possibly a very high content had caused stomatal opening after prolonged exposure, as found by Scarth (1932). It is conceivable that a humidity effect was concerned here, but *Pelargonium* stomata do not appear to close in response to dryness of the air-supply (p. 59); the very small corresponding effect with moist air in Expt. 71 may have been another example of the sluggish response of stomata under such conditions.

Another question which calls for further investigation is that of the validity of measurements of *Pelargonium* stomata by Lloyd's method. As discussed elsewhere (Heath, 1949, p. 191) it has been found that increased mean apertures by Lloyd's method do correspond to increased apertures on the living leaf as indicated by the porometer. For qualitative purposes, therefore, Lloyd's method may be used. In the present work, however, there is evidence that the stomata open during the processes of stripping and fixing (Section I). At the same time it seems very curious that in stomata freely exposed to the air *no* apertures could be seen by direct observation even after prolonged illumination with a 150-W. lamp at about 3 in. distance. Although stomata on the living leaf do not present a very favourable object, clear images were obtained, at least as good as the reproductions published elsewhere (Heath, 1938, pl. v, figs. 3 and 4), and if the stomata were in fact open the apertures must have been very small or they would have been resolved. There seems little doubt therefore that the apertures as seen by Lloyd's method were too large. It may be noted that the experiments in question were carried out at the end of September and beginning of October with 'Clone 5', in which at that time of year there is often a strong 'closing tendency' even for stomata enclosed in a porometer cup, especially in the afternoon.

4. *Biological importance of the carbon dioxide effect and humidity effects*

It might at first sight seem a curious adaptation that the stomata should close in response to increased carbon dioxide concentration when presumably their main function is to admit carbon dioxide to the leaf. When, however, the desiccating effects of wind are remembered, it seems likely that the sensitivity to carbon dioxide supply may serve the function of protecting the plant against excessive transpiration by causing partial stomatal closure in wind. Experiments shortly to be published from this Institute show that such stomatal control of transpiration in moving air does in fact occur, and this is also strongly suggested by the work of Wrenger (1935). In the case of wheat there is some evidence that this protective function of the carbon dioxide response is reinforced by a direct response to low atmospheric humidity, which causes closure either in still air (p. 43) or in moving air (p. 51). Except for the effect in darkness discussed earlier there is little suggestion of this in the *Pelargonium* data, e.g. the apparent slight *opening* caused by the change from still air to dry carbon dioxide-free air in Expt. 71 (p. 48). If this were a difference between an opening effect of carbon dioxide-free air and a closing effect of dry air, a much greater opening should have been shown with the moist carbon dioxide-free air, but in fact the opening here was negligible. The lack of appreciable response to changes in rate of flow shown in both dry and humid air in Expt. 71 also suggests that *Pelargonium* stomata have little tendency to close as a direct result of high evaporating power.

The *Pelargonium* results do, however, show a remarkable humidity effect on the *rate* of response to all the other stimuli investigated—carbon dioxide, light, and especially darkness; in each case the response is more rapid and equilibrium is attained sooner in dry air. In the case of light and dark this has also been found by Williams (1950). The possibility must be considered that this is in reality a carbon dioxide effect, any liquid water present acting as a reservoir of dissolved carbon dioxide: giving it out on illumination, absorbing it in darkness, and smoothing out effects of changes from moving ordinary air to carbon dioxide-free air and vice versa. This criticism might have cogency for Expts. 60 and 69, in which the well of the 'wet' cup was filled with wet broken porous tile, although even here there are strong indications that the *equilibria* attained were more widely separated for the 'dry' cup and the effect under discussion should leave the final equilibria unaltered. In Expt. 71, however, the well of the 'wet' cup was empty and any small quantity of moisture that might have condensed in it should have come quickly into equilibrium with the changed carbon dioxide contents. Moreover, the air moving at 4 l./hr. provided a complete change of the air in the cup about once every 1.8 seconds and it seems inconceivable that solution of carbon dioxide in moisture condensed on the walls should have had any appreciable effect. A curious example of the more rapid response in the 'dry' cups is provided by the effect of removing the glass plates. In Expts. 60 and 69 this caused much more rapid closure in the 'dry' than in the 'wet' cups, although the conditions

above the upper leaf surface were then identical and the presence or absence of the glass plates would be expected to affect mainly the upper stomata. It would not appear that solution of carbon dioxide in condensed moisture can possibly be concerned in this effect.

5. *The importance of equilibrium values in porometer experiments*

In connexion with the porometer experiments it has been mentioned that it was considered important to allow the readings to reach equilibrium under one set of conditions before changing to another: The reason for this is best seen by considering an example: supposing the stomata are still opening rapidly when a treatment which would normally cause closure is applied; the only effect may then be to reduce the rate of opening and stop it at a higher equilibrium resistance than would otherwise have been attained. There may not even be a very sharp bend in the curve at the point where the conditions were changed and the closing effect of the new treatment may thus be entirely overlooked. It is thought that this may have happened in earlier work with air passed through porometer cups between readings.

SUMMARY

It was found that stomata on illuminated leaves, both of *Pelargonium* and wheat, opened much wider where the leaf surface was enclosed in a small volume of air, as in a normal porometer cup, than elsewhere. This was shown for both species by the infiltration method, and for *Pelargonium* by Lloyd's method and direct microscopical observation also.

The effect was shown not to be due to pressure of the porometer cup or glass plate on the leaf, or to temperature differences, nor directly to the lack of movement or high humidity of the enclosed air.

A considerable body of data was collected which appeared to support the hypothesis that the wide opening was due to accumulation of some volatile substance produced by the leaf, but all the results were also consistent with the view that it was caused by reduction in the carbon dioxide content of the enclosed air below the normal 0.03 per cent. owing to photosynthesis. Further crucial experiments with both the porometer and infiltration methods left virtually no doubt that the latter hypothesis was correct.

This extreme sensitivity of stomata to carbon dioxide concentration within the range 0.03 per cent. to zero is discussed in relation to their operation in nature, and a possible biological advantage is suggested.

The bearing of the effect upon porometer investigations is also discussed and it is concluded that for all quantitative or semi-quantitative experimentation it is essential to use a cup detached between readings, or at least swept with air such as surrounds the rest of the leaf, and to have the upper leaf surface above the cup area freely exposed or similarly swept. For qualitative investigation of the light response of stomata the traditional form of cup may be used.

The importance is stressed of allowing porometer readings to reach equilibrium under one set of conditions before changing to another, when investigating the 'closing' or 'opening' effects of external factors.

Several subsidiary effects, observed in the course of the investigation, are discussed; in particular an effect of humidity upon the rate of response to other factors.

ACKNOWLEDGEMENTS

The writer wishes to thank Professor F. G. Gregory, F.R.S., for his constant and stimulating encouragement throughout this investigation; also Dr. W. T. Williams of Bedford College, who not only supplied the initial information from which it started but freely informed the writer of his further experimental results as they were obtained. Finally he wishes to express his gratitude to Dr. F. L. Milthorpe for valuable assistance on a number of occasions and for carrying out several experiments as noted in the text.

LITERATURE CITED

- BONNER, W., and BONNER, J. (1948). The rôle of carbon dioxide in acid formation by succulent plants. *Amer. J. Bot.* **35**, 113-17.
- DARWIN, F. (1898). Observations on stomata. *Phil. Trans. Roy. Soc., B*, **190**, 531-621.
- and PERTZ, D. F. M. (1911). On a new method of estimating the aperture of stomata. *Proc. Roy. Soc., B*, **84**, 136-54.
- FISHER, R. A., and YATES, F. (1948). *Statistical tables for biological, agricultural and medical research*. 2nd ed. London and Edinburgh. Oliver & Boyd.
- GREGORY, F. G., and ARMSTRONG, J. I. (1936). The diffusion porometer. *Proc. Roy. Soc., B*, **121**, 27-42.
- MILTHORPE, F. L., PEARSE, H. L., and SPENCER, H. J. (1950). Experimental studies of the factors controlling transpiration. I. Apparatus and experimental technique. *J. Exp. Bot.* **1**, 1-14.
- — — (1950a). Experimental studies of the factors controlling transpiration. II. The relation between transpiration rate and leaf water content. *Ibid.* 15-28.
- and PEARSE, H. L. (1934). The resistance porometer and its application to the study of stomatal movement. *Proc. Roy. Soc., B*, **114**, 477-93.
- — — (1937). The effect on the behaviour of stomata of alternating periods of light and darkness of short duration. *Ann. Bot., N.S.*, **1**, 3-10.
- HEATH, O. V. S. (1938). An experimental investigation of the mechanism of stomatal movement, with some preliminary observations upon the response of the guard cells to 'shock'. *New Phytol.* **37**, 385-95.
- (1939). Experimental studies of the relation between carbon assimilation and stomatal movement. I. Apparatus and technique. *Ann. Bot., N.S.*, **3**, 469-96.
- (1941). Experimental studies of the relation between carbon assimilation and stomatal movement. II. The use of the resistance porometer in estimating stomatal aperture and diffusive resistance. Part 1. A critical study of the resistance porometer. *Ibid.* **5**, 455-500.
- (1948). Studies in stomatal action. Control of stomatal movement by a reduction in the normal carbon dioxide content of the air. *Nature*, **161**, 179-81.
- (1949). Studies in stomatal behaviour. II. The rôle of starch in the light response of stomata. Part 1. Review of literature, and experiments on the relation between aperture and starch content in the stomata of *Pelargonium zonale*. *New Phytol.* **48**, 186-211.
- (1949a). Carbon dioxide in the intercellular spaces of leaves during photosynthesis. *Nature*, **164**, 822-3.
- and WILLIAMS, W. T. (1948). Studies in stomatal action. Adequacy of the porometer in the investigation of stomatal aperture. *Ibid.* **161**, 178-9.
- KNIGHT, R. C. (1915). A convenient modification of the porometer. *New Phytol.* **14**, 212-16.
- (1917). The interrelations of stomatal aperture, leaf water-content and transpiration rate. *Ann. Bot.* **31**, 221-40.

- KNIGHT R. C. (1922). Further observations on the transpiration, stomata, leaf water-content, and wilting of plants. *Ann. Bot.* **36**, 361–83.
- LINSBAUER, K. (1916). Beiträge zur Kenntnis der Spaltöffnungsbewegungen. *Flora*, N.F., **9**, 100–43.
- LLOYD, F. E. (1908). The physiology of stomata. *Carnegie Inst. Washington Publ. No.* 82, 1–142.
- MASKELL, E. J. (1928). Experimental researches on vegetable assimilation and respiration. XVIII. The relation between stomatal opening and assimilation. A critical study of assimilation rates and porometer rates in leaves of cherry laurel. *Proc. Roy. Soc., B*, **102**, 488–533.
- MOLISCH, H. (1912). Das Offen- und Geschlossensein der Spaltöffnungen, veranschaulicht durch eine neue Methode (Infiltrationsmethode). *Zeit. f. Bot.* **4**, 106–22.
- NEWTON, R. G. (1936). *The investigation of the relation between rates of assimilation and stomatal aperture in leaves*. Ph.D. Thesis. University of London.
- PENMAN, H. L. (1942). Theory of porometers used in the study of stomatal movements in leaves. *Proc. Roy. Soc., B*, **130**, 416–34.
- SAYRE, J. D. (1926). Physiology of stomata of *Rumex patientia*. *Ohio J. Sci.* **26**, 233–66.
- SCARTH, G. W. (1932). Mechanism of the action of light and other factors on stomatal behaviour. *Plant Physiol.* **7**, 481–504.
- SPENCER, H. J. (1938). *Factors concerned in the water relations of leaves*. Ph.D. Thesis, University of London.
- STAHL, E. (1919). Zur Physiologie und Biologie der Exkrete. *Flora*, N.F., **13**, 1–132.
- WILLIAMS, W. T. (1940). *A theoretical and experimental investigation of the factors concerned in stomatal movement with special reference to the transmission of shock stimuli*. Ph.D. Thesis, University of London.
- (1947). Shock-induced stomatal movements. *Nature*, **160**, 364–5.
- (1949). Studies in stomatal behaviour. III. The sensitivity of stomata to mechanical shock. *Ann. Bot.*, N.S., **13**, 309–27.
- (1950). Studies in stomatal behaviour. IV. The water-relations of the epidermis. *J. Exp. Bot.* **1**, 114–31.
- WRENGER, M. (1935). Über den Einfluss des Windes auf die Transpiration der Pflanzen. *Zeit. f. Bot.* **29**, 257–320.

The Path of Carbon in Photosynthesis

VII. RESPIRATION AND PHOTOSYNTHESIS¹

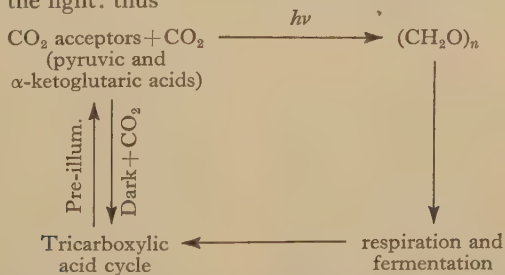
A. A. BENSON AND M. CALVIN

The Radiation Laboratory, Department of Chemistry, University of California, Berkeley, California

PREVIOUS work (Calvin and Benson, 1948; Benson, Calvin, *et al.*, 1949) has shown that illumination of an algal suspension in the absence of carbon dioxide greatly enhances its ability to fix carbon dioxide in an immediately following dark period. The kinetics of the generation and decay of this ability have been determined, and it was shown that less than one minute of illumination was sufficient to bring this fixing ability almost to its saturation value.

Other experiments (Benson and Calvin, 1948) which determined the dependence of dark fixation rate on carbon dioxide pressure, showing it to be very similar to, if not identical with, the dependence of steady state photosynthesis upon carbon dioxide pressure, were used as a partial argument in support of the suggestion that the enhanced dark fixation immediately following illumination in the absence of carbon dioxide was indeed the process of carbon dioxide reduction taking place in photosynthesis.

Nevertheless, kinetic arguments involving unknown chemical reactions being what they are, it was still conceivably possible that this enhanced dark fixation was due only to the reversibility of the respiratory and fermentative decarboxylations. The photosynthesis occurring during pre-illumination presumably reduced the carbon dioxide partial pressure by reactions as yet unknown, thus shifting the fermentative and respiratory reaction equilibria in the direction of decarboxylation. Upon the introduction of carbon dioxide in the dark, the equilibria are shifted in the opposite direction, thus giving the enhanced fixation, but by processes presumed to be quite different from those taking place in the light: thus



If this be the case, then the products formed in dark fixation of carbon dioxide should be the same following pre-illumination in the absence of carbon dioxide as they are following a dark saturation with carbon dioxide, and quite different from those formed in the light. If the pre-illuminated dark

¹ This work was supported by the United States Atomic Energy Commission.

← Water-saturated phenol.

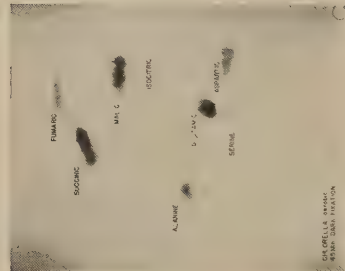
Butanol-Water-Propionic acid. →



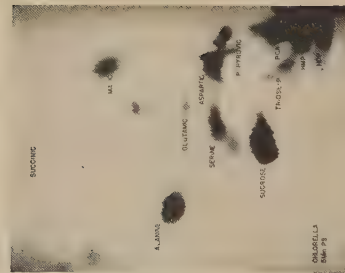
30 sec. PS. *Chlorella*.



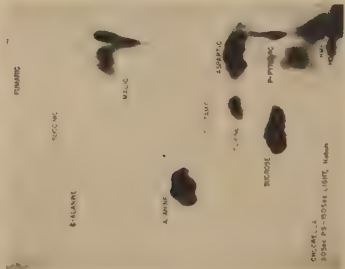
2 min. dark pre-illuminated *Chlorella*.



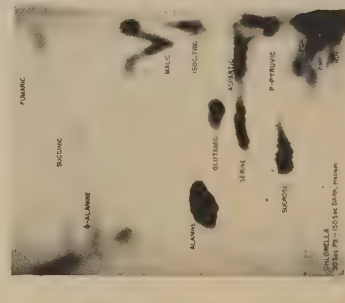
45 min. dark *Chlorella*.



5 min. PS. *Chlorella*.



30 sec. PS.-15 sec. light (He) *Chlorella*.



30 sec. PS.-15 sec. dark (He) *Chlorella*.



30 sec. PS.-150 sec. light *Chlorella*.



30 sec. PS.-150 sec. light *Scenedesmus*.

Butanol-water-propionic acid —→

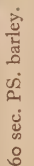


FIG. 1. C^{14} Radiograms of extracts of plants exposed to $C^{14}O_2$ under selected conditions. (For meaning of symbols, see Table I.)

fixation were the same process that takes place in ordinary photosynthesis, the products formed should be the same as those found in an equivalent period in the light and different from those formed in the dark without pre-illumination. If some of the compounds or closely related substances which appear in respiration and fermentation are also intermediates in photosynthesis, they should be formed in all three cases.

Figure 1 is a set of radiograms showing the products formed in each of the three cases for algae and for barley leaves (see 30 s. PS.C., 2 m. D.PI.C., 45 m. D.C., 30 s. PS.B., 2 m. D.PI.B., 50 m. D.B. in Table I). It is apparent that the products formed in dark fixation following pre-illumination in the absence of carbon dioxide correspond very closely to those formed in direct photosynthesis and not to all of those formed in dark fixation following dark saturation with carbon dioxide. These latter are indeed the substances expected from the simple reversibility of respiratory and fermentative reactions. We can thus confirm the suggestion, at first made on the basis of kinetic studies (Benson and Calvin, 1948), that all of the reactions lying between carbon dioxide and sucrose are dark reactions. The reducing energy required to achieve this transformation is supplied by the photochemical reaction involving the photolysis of water. Furthermore, the reducing power so provided is in the form of a definite chemical species rather than in the form of some excited electronic state of a molecule. This conclusion has been reached previously as a result of studies of fluorescence (Wassink and Katz, 1939) and of the comparative biochemistry of certain bacteria (Van Niel, 1941). The continued carbon dioxide absorption in the dark immediately following a strong illumination (McAlister and Myers, 1940; Emerson and Lewis, 1941) constitutes the direct observation of the effect. It is of interest to note that the successful separation of the photochemical apparatus for the splitting of water and the evolution of oxygen (Hill, 1937, 1939, Hill and Scarisbrick, 1940) from the carbon dioxide reducing system has been accomplished.

Figure 1 shows that a number of radioactive compounds are common to all three of the radiograms. These are, particularly, alanine, serine, aspartic acid, and malic acid. They may be taken as indicators for the presence of the corresponding keto-acids, pyruvic and oxalo-acetic acids, under all three circumstances. It is thus apparent that some compounds are involved as intermediates in both the photosynthetic and the respiratory cycles. Whether the common reservoirs of the two cycles are identical or are physically separate in the organism remains to be determined.

A number of experiments have been done in an attempt to determine something about the relationship between the photosynthetic and respiratory paths. A simple design for such an experiment would be to introduce the label into some of the photosynthetic intermediates by a short period of photosynthesis in radioactive carbon dioxide¹ and follow this by a suitable period of respiration in either dark or light. The result of such an experiment on algae is shown

¹ The experimental methods have been described previously: M. Calvin and A. A. Benson (1949); A. A. Benson *et al.*, *J. Amer. Chem. Soc.* (in press).

TABLE I

Percentage distribution of radioactivity among aqueous alcohol-soluble non-lipid products

	Alanine	Aspartic	Glutamic	Glutamine	Glycine	Serine	Succinic	Fumaric	Glycolic	Isocitric (citric?)	Malic	Sucrose	Phosphate esters
30 s. PS.C. ^a	6.1	0.8	—	—	—	1.3	—	—	0.5	—	0.9	0.5	89 ^l
2 m. D.PI.C. ^a	39	5.5	0.4	—	—	7.3	0.4	—	—	0.2	2.4	6.7	38
45 m. D.C. ^a	11	8.7	23	1.5	—	4.0	17	7.5	—	1.9	21	1.2	1.2
30 s. PS.B. ^b	1.1	0.1	—	—	12	4.9	—	—	8.3	—	0.5	15	59 ^m
2 m. D.PI.B. ^a	33	3.4	1.2	—	—	2.2	—	—	—	—	4.9	1.9	53 ⁿ
50 m. D.B. ^a	2.2	28 ²	17	8.2	—	4.7	—	—	—	6	31	—	2.9
30 s. PS.—150 s. L.(He)C. ^c	23	17	0.4	—	—	3.8	—	—	—	—	8.4	27	21 ^p
30 s. PS.—150 s. D.(He) ^d C.	27	3.6	3.1	—	—	4.5	—	0.9	—	0.5	3.4	7.2	50 ^q
30 s. PS.—150 s. L.C. ^e	8.0	6.1	0.5	—	4.1	8.3	—	—	6.7	0.6	6.7	31	25 ^r
30 s. PS.—150 s. L.Sc. ^f	1.2	3.9	1.5	—	0.9	3.7	—	—	3.7	2.2	25	1.9	56 ^s
5 m. PS.C. ^g	1.9	3.0	0.4	—	—	2.0	0.2	—	—	0.1	1.9	23	65 ^t
30 s. PS.—2 m. L.B. ^h	2.2	1.4	—	—	7.4	6.7	—	—	23	—	2.3	45	11 ^u
30 s. PS.—2 m. D.B. ^b	4.6	0.8	—	—	0.7	17	—	—	0.2	0.5	0.7	34	42 ^v
30 s. PS.—40 m. D.B. ^h	6.4	1.6	2.3	1.4	—	7.3	—	—	—	0.8	2.1	62	16 ^w
60 s. PS.B. ^j	7.7	2.6	—	—	3.3	3.7	—	—	2.0	—	3.3	27	50 ^x
5 m. PS.B. ^k	8.1	0.7	0.8	—	4.5	4.3	—	—	6.4	0.2	2.6	65	6 ^y

Percentages were determined by direct counting of areas on the paper chromatogram defined by the radiogram.

s., seconds; m., minutes; PS., photosynthesis with C¹⁴O₂; C., *Chlorella pyrenoidosa*; Sc., *Scenedesmus D 3*; L., light; D., dark; B., barley seedling leaves; PI., 5–10 min. pre-illumination in helium; He, helium.

All treatments subsequent to photosynthesis were done in CO₂-free air unless specified as in helium.

^a In these experiments less than 5% of the total radioactivity fixed was insoluble in alcohol-water. ^b 2.5% insol. ^c 25% insol. ^d 6% insol. ^e 30% insol. ^f 30% insol. ^g 45% insol. ^h 10% insol. ⁱ 6% insol. ^j 3.2% insol. ^k 4.5% insol. ^l includes: Phosphoglyceric acid 24% (PGA), Hexose monophosphates 60% (HMP), Hexose diphosphates 3.9% (HDP), Triose-phosphates 0.5% (TriP). ^m includes free glyceric acid 10%. ⁿ includes free glyceric acid 2%. ^p includes PGA 1.3%, HMP 14%, HDP 4%, P. Pyruvic 1.8%. ^q includes PGA 6%, HMP 35%, HDP 7%, P. Pyruvic 1.8%. ^r includes PGA 3.2%, HMP 13%, HDP 7%. ^s includes PGA 25%, HMP 11%, HDP 18%, P. Pyruvic 2.2%. ^t includes PGA 26%, HMP and HDP 39%, P. Pyruvic 0.6%. ^u includes free glyceric acid 0.7%. ^v includes free glyceric acid 15%. ^w includes free glyceric acid 9%. ^x includes free glyceric acid 6%. ^y includes free glyceric acid 0.6%. ^z includes asparagine 2%.

in Fig. 1. One effect is immediately apparent. In those cases in which the algae have not been given a dark period either during or after exposure to C*O₂ there is little or no labelled glutamic and isocitric acid formed (see 30 s. PS.C., 30 s. PS.—150 s. L.(He)C., and 5 m. PS.C., Table I). If, however, they are given as little as 150 sec. of dark time following 30 sec. of photosynthesis in C*O₂, relatively large amounts of labelled glutamic and isocitric acids appear (see 30 s. PS.—150 s. D.(He)C., Table I). If the dark period is aerobic, the amounts of these compounds which appear are somewhat larger than under anaerobic conditions. It is thus clear that the light not only initiates a series of reactions constituting the photosynthetic reduction of carbon

dioxide but also in some way inhibits certain other reactions. Although the nature of the inhibition is as yet unknown, it is clear that light prevents some of the newly formed photosynthetic intermediates from participating in the tricarboxylic acid cycle as represented by glutamic and isocitric acids. In the barley leaves the formation of labelled glutamic acid is so slight even after relatively long dark periods that we cannot yet say whether the same phenomenon occurs or not. It is indicated by a comparison of the isocitric contents of 30 s. PS.B., 5 m. PS.B., 30 s. PS.-2 m. L.B., and 30 s. PS.-2 m. D.B., in which the last contains more of this acid than any of the first three. In the barley experiments another effect can be seen, which involves the conditions under which labelled glycine and glycolic acid are found. Glycine and glycolic acid, formed in the light from radiocarbon dioxide, disappear in as little as 2 min. in the dark. They appear in detectable amounts in as short a period as 5 sec. of photosynthesis in radiocarbon dioxide and are maintained in the light in the absence of carbon dioxide with the glycolic acid increasing (see 30 s. PS.B., 30 s. PS.-2 m. D.B., and 30 s.-2 m. L.B.). The same effect has been observed with algae. Additional experiments with algae showed that the presence of oxygen during the subsequent illumination enhanced the formation of labelled glycine and glycolic acid. In the case of glycine, for which the total amount present may be estimated from the amount of the ninhydrin colour produced on the paper, it appears that the short dark periods reduce not only the amount of labelled glycine but the total glycine as well. This would seem to indicate that the size of the reservoirs of free glycine and presumably of glycolic acid vary considerably with illumination and may possibly be related to the photosynthetic path of carbon. Degradation studies now under way should lead to a detailed exposition of the sequence of compounds involved.

LITERATURE CITED

- BENSON, A. A., and CALVIN, M. (1948). The path of carbon in photosynthesis. III. *Cold Spring Harbour symposia on quantitative biology*, **13**, 6.
- BENSON, A. A., CALVIN, M., *et al.* (1949). C¹⁴ in photosynthesis. Chapter 19, *Photosynthesis in plants*. Iowa State College Press.
- BENSON, A. A., *et al.* The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. *J. Amer. Chem. Soc.* (in press).
- CALVIN, M., and BENSON, A. A. (1948). The path of carbon in photosynthesis. I. *Science*, **107**, 476.
- (1949). The path of carbon in photosynthesis. II. Intermediates in sucrose synthesis. *Science*, **109**, 140.
- EMERSON, R., and LEWIS, C. M. (1941). Carbon dioxide exchange and the measurement of quantum yield of photosynthesis. *Amer. J. Botany*, **28**, 789.
- HILL, R. (1937). Oxygen evolved by isolated chloroplasts. *Nature*, **139**, 881.
- (1939). Oxygen produced by isolated chloroplasts. *Proc. Roy. Soc. (Lond.)*, *B*, **127**, 192.
- and SCARISBRICK, R. (1940). Production of oxygen by illuminated chloroplasts. *Nature*, **146**, 61.
- MCALISTER, E. D., and MYERS, J. (1940). The time course of photosynthesis and fluorescence. *Smithsonian Inst. Publ. (Misc. Coll.)*, **99**, No. 6.
- VAN NIEL, C. B. (1941). The bacterial photosyntheses and their importance for the general problem of photosynthesis. *Adv. Enzymology*, **1**, 263.
- WASSINK, E. C., and KATZ, E. (1939). The initial changes in chlorophyll fluorescence in *Chlorella*. *Enzymologia*, **6**, 145.

Demonstration of Compound Cilia in a Fern Spermatozoid by Means of the Ultra-violet Microscope

I. MANTON

Botany Department, University of Leeds

WITH 3 PLATES

THE observation contained in Figs. 1-7 was obtained incidentally whilst using fern spermatozoids for other purposes. It is, however, perhaps of sufficient interest to justify separate publication, since it could scarcely have been obtained by any ordinary visual means.

The material was not specially selected for the purpose beyond being from a healthy culture of young prothalli which happened to be in use for the synthesis of certain fern hybrids. In the present context its specific identity is probably immaterial, though it may be described as that of a diploid form of *Dryopteris Villarsii* Woynar, native to the island of Cyprus.

The technical treatment given to the preparation of the specimen for ultra-violet microscopy was of the simplest. The spermatozoids were induced to swim by submerging some prothalli carrying antheridia for a few minutes in water in a watchglass. As soon as a sufficient number could be seen in motion a drop of the water containing them was put on to a quartz slide with a pipette. The spermatozoids were killed by 30 seconds' exposure to osmic vapour, after which a small amount of iodine in potassium iodide was added to the drop to make them more easily visible under the ordinary microscope. A quartz coverslip was put on and ringed with wax. A suitable cell was then selected under the visual-light microscope and examined in detail by serial photography on the ultra-violet microscope. One sample focal level through an unusually beautiful specimen is shown in Fig. 1. The uncoiled position of the curved nucleus is an artifact which occurred as the cell was killed. In all other respects it appears to be in a very natural condition.

As a control, another preparation was made in the same way except that it was allowed to dry between the killing of the spermatozoid and the addition of iodine, thereby forcing all parts of the cell into approximately one plane on the surface of the slide and preventing to some extent the loss of cilia, which are otherwise liable to become detached from the body. Fig. 2 is a photograph of a specimen so obtained. It shows the spirally coiled nucleus somewhat disarranged and about fifty whip-like cilia, each tapering to an imperceptible point at the end.

That this, the conventional, appearance of the cilia is an incomplete picture is, however, revealed by the details of Figs. 1 and 2 and by the enlarged portions of these contained in Figs. 3 and 4. In many parts of both Figs. 1 and 4

the whip-like cilia are represented by bundles of delicate threads, each so fine that its width is almost certainly below the limit of true resolution even of the ultra-violet microscope, though the length enables them to be seen. That they are genuine structures and not a diffraction pattern or 'flare' caused by cilia out of focus is proved, among other reasons, by their attitudes; in many places they diverge widely at the end of a bundle, an appearance which contrasts totally with the strongly marked parallel lines that a cilium genuinely out of focus actually displays in other parts of the same photographs.

Lest, however, the clarity of this observation should become obscured by mechanical reproduction of photographs in half-tone, Figs. 5-7 have been added. In these the intensity of the lines has been enhanced by photographic means. The resulting pictures are harsh and unpleasing, but the critical details are perhaps more clearly displayed. In them the number of threads composing each bundle can be roughly assessed as of the order of ten.

As far as I am aware this is the first demonstration of compound cilia in plants, though compound cilia in *Paramoecium* were demonstrated with the electron microscope in 1946 by Jakus and Hall.

The ultra-violet microscope used for this work is the newly designed Cooke, Troughton and Simms instrument recently erected in the Botany Department at Leeds with the aid of a grant from the Royal Society. It carries a Zeiss 1.7-mm. glycerine immersion quartz lens computed to work with the 2,750 Å Cadmium line supplied by a stationary electrode spark.

I am greatly indebted to my technical assistant, Mr. B. Clarke, for the care and skill with which the photographs have been prepared and for help in many other ways.

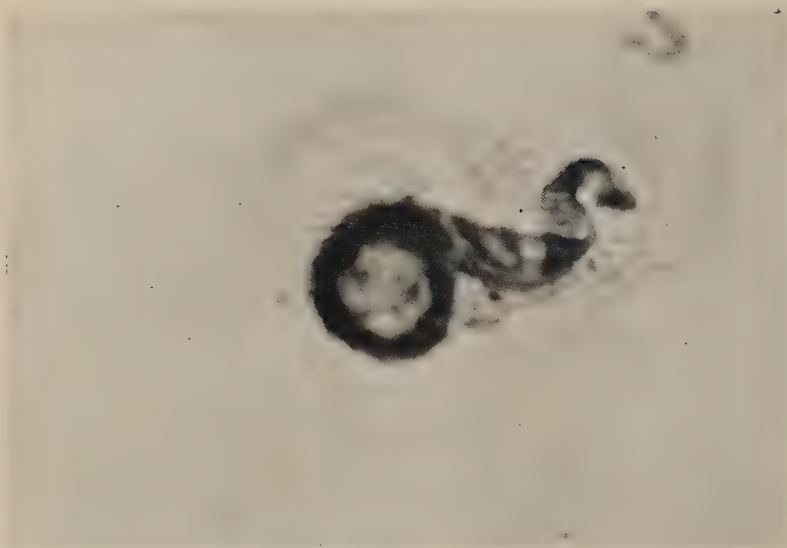
LITERATURE CITED

- JAKUS, M. A., and HALL, C. E. (1946). Electron microscope observations of the trichocysts and cilia of *Paramoecium*. *Biological Bulletin*, **91**, 141-4.



FIG. 1. High-power U.V. photograph of a fern spermatozoid killed with osmic vapour and mounted in iodine. Magnification $\times 3000$

I. MANTON—PLATE I



3



4

FIG. 2. The same as figure 1 but air-dried on to the slide before examination in iodine.

$\times 3000$

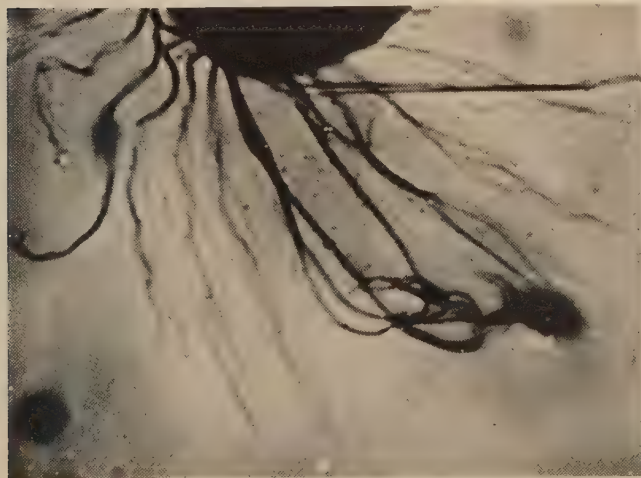
FIG. 3. Enlarged detail of a portion of figure 2. $\times 4000$

FIG. 4. Enlarged detail of a portion of figure 1. $\times 4000$

5



6



7

FIGS. 5-7. Enlarged details of portions of figure 1, $\times 4000$,
after photographic intensification

I. MANTON—PLATE III

Further Studies on the Acid Metabolism of *Aspergillus niger*

THE FORMATION AND UTILIZATION OF OXALIC ACID

A. ALLSOPP

Department of Cryptogamic Botany, The University, Manchester

INTRODUCTION

DURING the past decade it has become increasingly apparent that the organic acids play an important role in the metabolism of a wide range of organisms. But although the findings and hypotheses of many investigators have contributed greatly to our knowledge and understanding of the conversions and functions of the familiar tricarboxylic and 4-C dicarboxylic acids, the metabolism of oxalic acid has received very little attention. This neglect is perhaps hardly surprising when it is considered that the majority of workers have confined their attention to animal tissues or bacteria, where oxalic acid is scanty or lacking and is usually said to play little or no part in metabolism.

The slight consideration afforded to oxalic acid in recent years by those investigating the metabolism of plants is less understandable, in view of the fact that this acid is present, at least as small quantities of calcium oxalate crystals, in virtually all green plants, certain of which contain considerable amounts of free acid, which is also produced in quantity by many fungi. Many strains of *Aspergillus niger* are particularly active in the formation of oxalic acid and have received most attention from workers in this field. It seems not unlikely that results obtained by investigations of this and other fungi may be of considerable value in the interpretation of the metabolism of the higher plants.

By contrast with the bulk of work on fungal metabolism, the majority of metabolic studies have been carried out using minced tissues, tissue slices, tissue extracts, or even isolated enzymes. Studies of this type are necessary in unravelling the complicated systems of reactions occurring in living tissues, and could probably be applied with great advantage to the investigation of the fungi, but the value of such studies is at least doubtful unless they are supported by complementary studies on living organisms. This approach has been stressed by Nord and Mull (1945) and by Leibowitz and Hestrin (1945).

Many fungi provide an almost ideal system for the study of the metabolism of living tissues, for they may be maintained on an extensive range of liquid media without any evident disturbances of the normal life processes. The use of living cultures, however, introduces certain dangers in interpretation and many workers on acid metabolism appear to have overlooked some of the potentialities of the active living fungus. The complexities involved in the

apparently simple formation of oxalic acid from a single substrate are indicated in the present contribution, which is concerned principally with the effect of glucose concentration on the formation and utilization of oxalic acid and the influence of added oxalic acid on acid formation and CO_2 output.

The earlier work on the formation of oxalic acid by *Aspergillus niger* has already been summarized by the present writer (Allsopp, 1937). Most of the literature deals with attempts to elucidate the chemical mechanism of the formation of this acid. The widely divergent views held by different schools suggests either a surprising complexity in the mechanism of the processes or a fundamental error in the interpretation of results. The second explanation is certainly in part responsible. Hypotheses of oxalic acid formation have usually been based on feeding experiments, in which it is assumed that any oxalic acid appearing in the external medium is derived by conversion of the substance supplied. Absence of oxalic acid is taken to indicate that the tested substance plays no part as an intermediate product.

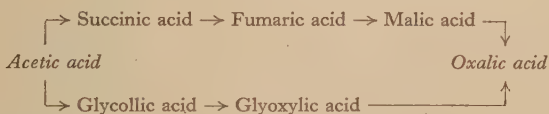
Using this approach various workers have obtained oxalic acid from an extensive range of substrates of widely varying structure. In the view of the writer this is not an unexpected result and bears testimony to the pronounced synthetic powers of the fungus rather than to the origin of oxalic acid by direct oxidation of the substrate. It would appear that in many instances the supplied substance is converted into reserve substances of the mycelium, the oxalic acid arising directly from the carbohydrates of the latter. This view seems particularly probable when applied to experiments in which salts of organic acids are supplied as possible intermediates. With such salts as the sole source of carbon, growth of the mycelium must involve utilization of the organic anions, leaving an excess of metallic cations, which are known to trap oxalic acid, inhibiting its further conversion.

This and other aspects are discussed in detail by Bennet-Clark (1933), Bennet-Clark and La Touche (1935), and in the author's earlier paper (Allsopp, 1937), but in view of certain recent contributions it has been considered desirable to restate this point of view. Thus, as a result of his fairly extensive researches, Jacquot (1938 *a, b, c*) concluded that oxalic acid fermentation is an occasional phenomenon characteristic of certain conditions of culture. On the basis of a limited number of experiments, in which oxalic acid was added to chopped mycelia mixed into a homogeneous mass, he concluded that the acid constitutes an ultimate product of metabolism and that oxalic acid formation is not a respiratory phenomenon of which the ultimate products are CO_2 and water. It is not proposed to discuss Jacquot's results in further detail since it is shown in the present and in earlier work (Allsopp, 1937) that oxalic acid undergoes further conversion when supplied to our strain of *A. niger*.

The formation of oxalic acid by other fungi has also received attention in recent years. In *Sclerotium rolfsii* Sacc. Perlmann (1948) obtained high yields of oxalic acid in the presence of calcium carbonate as trap. As in *A. niger* the acid is produced from a large number of sources and there is apparently no

structural relationship between the substrate supplied and the oxalic acid produced. The present writer is in agreement with Perlmann when he concludes that oxalic acid is not always a direct dissimilation product of the carbon substrate. It was also clearly shown that the fungus utilizes oxalic acid, particularly in the presence of an iron salt.

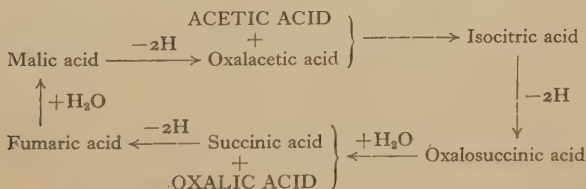
Nord and Vitucci (1947, 1948) devote a section of their interesting studies on the biochemistry of wood-destroying fungi to a consideration of oxalic acid formation. For this aspect of the work *Merulius niveus*, *M. tremellosus*, *M. confluens*, and *Fomes annosus* were the fungi used. Since these organisms produce ethyl alcohol, acetaldehyde, acetic acid, and succinic acid when reared on glucose as substrate it was suggested that oxalic acid might arise from acetic acid by either of the pathways of the following scheme:



After incubation periods of the order of 20–48 days oxalic acid was found in quantity in cultures reared on the salts of all the acids represented in the above scheme. From these results the authors conclude that oxalic acid formation proceeds along either of the two pathways indicated above. In the view of the present writer, however, although these pathways are not inherently improbable, the evidence is insufficient to demonstrate their occurrence. For reasons discussed above, oxalic acid would be expected to accumulate in any culture where utilization of an organic anion results in an increase in the alkalinity of the medium. This criticism is upheld by the fact that the authors obtained oxalic acid from all the acids supplied including pyruvic, lactic, dimethyl succinic, and dimethyl fumaric acids.

The views of Nord and Vitucci are supported by Smith (1949), who studied oxalic acid formation in *Merulius lacrymans* and *Marasmius chordalis*. The formation of glyoxylic acid by the former was accepted as evidence in favour of the suggested scheme.

Lynen and Lynen (1948), however, in their carefully considered work on oxalic acid formation by *A. niger*, suggest that glyoxylic acid is produced by the reduction of oxalic acid. They propose the following cycle to account for the conversion of acetic acid to oxalic acid:



Hamada (1940) in his studies on *Armillaria mellea* has given some consideration to the significance of oxalic acid formation, but in the main his views are based merely upon the presence or absence of crystals of calcium oxalate. He groups *A. mellea* with those fungi, 'N-Bevorzuger', which are said to form oxalic acid only by the deamination of various amino compounds such as peptone, by contrast with the 'C-Bevorzuger', such as *Aspergillus niger*, which also form oxalic acid from various C sources. Both processes are considered to be essentially similar in that acid is only produced from that substance which for various reasons cannot be used in growth. Oxalic acid formation is thus considered to be a superfluous additional activity of the fungus.

From the survey of certain recent contributions it will be apparent that fundamentally divergent views are held as to the significance of oxalic acid in the metabolism of the fungi. It may also be concluded that in the present inadequate state of our knowledge of the conditions governing the accumulation and further utilization of the acid, any hypotheses as to the chemical mechanism of the process must be treated with the greatest caution if based merely on the supply of hypothetical intermediates to the living fungus. In the present work certain conditions influencing the formation and utilization of oxalic acid are examined.

EXPERIMENTAL METHODS

The methods used were closely similar to those already described (Allsopp, 1937). As in the earlier work all cultures were prepared from a single strain of *A. niger* catalogued as S.I. 133C.

Standard conditions were adopted in which the fungus was grown on 250 ml. of the modified Czapek-Dox synthetic medium in 500-ml. bolt-head flasks. The mats of uniform area prepared in this way, using a dense sowing of spores, were closely similar in behaviour as checked by CO₂ output and acid yield. After a suitable period of growth the culture solution was replaced by sterile water for several days, and following this short period of starvation the experimental solutions were supplied. All operations were carried out under sterile conditions.

As described in detail in the previous publication, the culture solutions were stirred by the entering stream of CO₂-free air. The CO₂ output was determined by absorption in Pettenkofer tubes containing caustic soda solution. Samples of the supplied solutions were removed at daily intervals. Titratable acidity was determined by titration with 0.02 N carbonate-free sodium hydroxide solution. Any oxalate in these samples was determined as calcium oxalate after precipitation with Bau's reagent. Glucose was determined by the micro-carbonate-tartrate reagent of Shaffer and Hartmann.

Many of the data obtained are considered only in outline in the following account, but complete records are available for reference if required. Each culture is designated by a letter followed by a numeral. Cultures with the same letter were prepared from the same subculture. The numeral identifies the individual culture.

EXPERIMENTAL RESULTS

The formation of oxalic acid from glucose

In previous work (Allsopp, 1937) it was found that when solutions of pure glucose were supplied to this strain of *A. niger* the concentration of oxalic acid increased rapidly until a certain level was attained, after which there was no further accumulation of acid although glucose was still present in considerable amounts. It was further shown that if a culture were supplied with a solution of glucose+oxalic acid, with the concentration of the latter slightly above that normally attained on glucose only, there was no increase in oxalic acid concentration, but instead a decrease until the normal level was reached. From these results it was suggested that there is an equilibrium concentration of oxalic acid which is not exceeded, the equilibrium level being maintained either by a conversion of the acid into CO_2 or its resynthesis into some other compound, possibly carbohydrate.

The present work was designed to test this conception of oxalic acid accumulation, and to supply further information of the conditions governing this equilibrium.

Section (a). The Effect of Glucose Concentration

Group N. Cultures N1, 10, 6, and 4 were prepared on the standard medium and after 14 days of growth the mycelia were washed and supplied with 250 ml. of water. Following 2 days of starvation the cultures were supplied with 18.6, 9.5, 4.7, and 0.86 per cent. solutions of glucose respectively.

In planning this experiment it was thought that oxalic acid concentration might be maintained at the same level on different glucose concentrations. Such a result would be expected if oxalic acid were produced by the fungus to create the conditions most favourable for its growth, &c.

Instead of the expected result, it was found that the equilibrium level of oxalic acid diminished with increasing glucose concentration, while the rate of acid accumulation was also lower at the higher glucose concentrations.

These results are not published in detail since a more complete record is available for the next group of cultures.

Group S. Cultures S1, 2, 4, and 5 were reared on 250 ml. of the standard medium for 7 days, starved on tap-water for 2 days, and on the 9th day of growth received 250 ml. of approximately 1, 5, 10, and 20 per cent. glucose solutions.

The values for titratable acidity are set out in Fig. 1. The acid concentration of the culture S1 is at first in excess of that of the other cultures, but coinciding with the depletion of the glucose supply is a rapid fall in acid content. In the remaining cultures there is a diminution in the maximum acid concentration with increasing concentration of glucose.

Oxalate determinations revealed that the acid is virtually pure oxalic acid

in culture S₁, in S₂ and S₄ there is a small proportion of another acid, while in S₅ the undetermined acid rises to about 50 per cent.

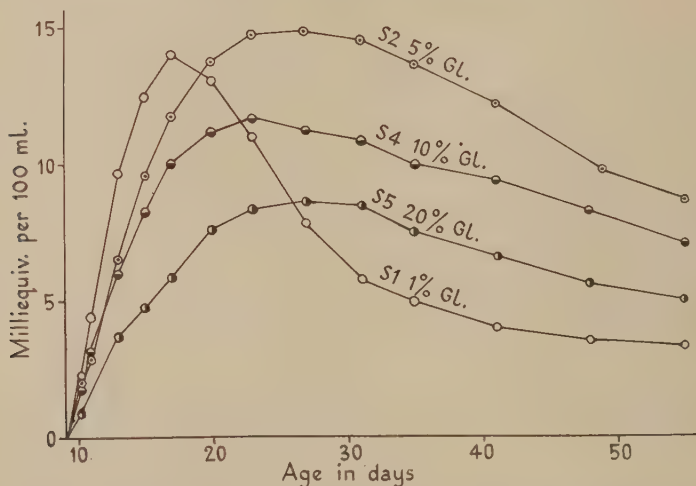


FIG. 1. Acid formation by cultures S₁ (—○— 1% glucose), S₂ (—●— 5% glucose), S₄ (—●— 10% glucose), and S₅ (—●— 20% glucose). The results are expressed as milliequivalents of titratable acid per 100 ml. culture solution.

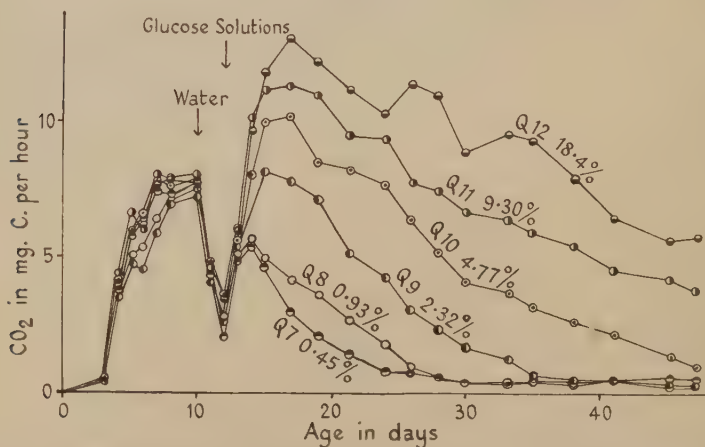


FIG. 2. CO₂ production by cultures Q₇ (—●—), Q₈ (—○—), Q₉ (—●—), Q₁₀ (—●—), Q₁₁ (—●—), and Q₁₂ (—●—). On the 12th day the cultures were supplied with 0.45, 0.93, 2.32, 4.77, 9.30, and 18.4% solutions of glucose respectively.

Group Q (a). Cultures Q₇, 8, 9, 10, 11, and 12 were reared under standard conditions for 10 days, starved on tap-water for 2 days, and on the 12th day of growth supplied with 0.45, 0.93, 2.32, 4.77, 9.30, and 18.4 per cent. solutions

of glucose respectively. The rates of CO_2 output are set out together in Fig. 2 and the acid concentrations in Fig. 3.

A consideration of the CO_2 output shows a close correspondence between the cultures during the first 12 days of growth. Starvation results in a steep fall in CO_2 output, while in every case the addition of sugar is followed by an increase. The sugar concentration has a marked effect on the CO_2 output which increases with increase in glucose concentration.

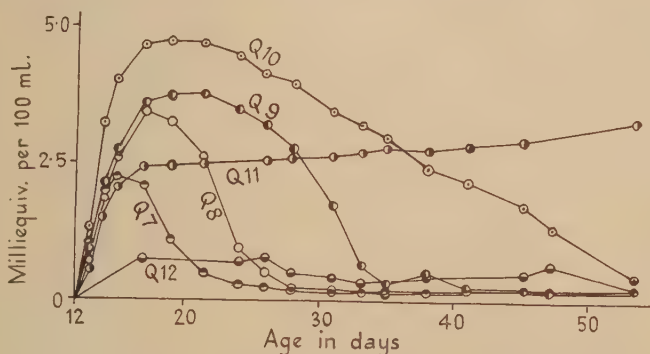


FIG. 3. Acid formation by cultures Q7 (—●—), Q8 (—○—), Q9 (—○—), Q10 (—○—), Q11 (—○—), Q12 (—○—). The values for Q7, 8, 9, 10, and 11 represent titratable acid, for Q12 oxalic acid. On the 12th day the cultures were supplied with 0.45, 0.93, 2.32, 4.77, 9.30, and 18.4% solutions of glucose respectively.

The acidity curves are no less striking. It was expected from the results of Groups N and S that there would be a continuous falling off in the rate of oxalic acid formation with increasing glucose concentration. This is true for the higher glucose concentrations in Q10, 11, and 12, but from Q10 down to Q7 there is a continuous diminution in the maximum content of oxalic acid. A closer examination of the data, however, shows that the results are not opposed to those expected. A comparison with Group N reveals that the cultures of Group Q are far more active, as shown by their higher rate of CO_2 output and especially by their rapid rate of glucose utilization. As a result of this high rate of sugar depletion, in the cultures supplied with low concentrations of glucose, the glucose has disappeared before the maximum oxalic acid concentration has been attained. Such a reduction to a low glucose concentration is invariably followed by a steep fall in the concentration of oxalic acid. When glucose is present in a concentration sufficient to permit the attainment of an equilibrium level, a characteristic curve is obtained showing a steady fall with age, as in Q10, and from this point the oxalic acid concentration diminishes with increase in glucose concentration.

In this group, except in Q12, oxalate determinations were carried out only on isolated occasions. The results show that in Q12 oxalic acid forms only a small proportion of the titratable acid, in Q11 there is a smaller proportion of undetermined acid, while the other cultures produce mainly oxalic acid.

The cultures of Groups N, Q, and S were prepared from subcultures of the same strain of *A. niger* but show considerable differences in the rates of glucose utilization, CO₂ output, acid formation, &c. The maximum acid concentration of S > N > Q, while the rates of CO₂ output show a reverse progression.

A high acid concentration appears to be associated with a slow rate of glucose utilization, since glucose disappeared slowly from cultures of Groups N and S, but rapidly from those of Group Q.

Group Q (b). It has been suggested by several workers, notably by Hamada (1940), that oxalic acid formation results from a conversion of materials present in excess which cannot be utilized in the ordinary growth processes.

Since the results already described in the present paper were obtained by supplying various glucose concentrations to preformed mycelia, it seemed desirable to investigate the effect of various glucose concentrations when supplied together with the full complement of mineral salts from the time of inoculation.

Cultures Q1, 2, 3, 4, 5, and 6 were grown under standard conditions on 250 ml. of the normal medium, but with the usual 5 per cent. glucose concentration replaced by concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 per cent. respectively. As in the cultures of Group Q (*a*), the CO₂ output increased with increasing glucose concentration.

The titratable acid values were also similar to those of Group Q (*a*) in that the maximum acid concentration was lower at the higher glucose concentrations. The acid values rose sharply during the period of rapid growth, without the considerable lag that might be expected if the views of Hamada are valid.

Section (b). The effect of oxalic acid

In previous work (Allsopp, 1937) it was suggested that in the presence of excess glucose there was a tendency for the oxalic acid concentration to remain at a more or less constant level which was maintained by the further conversion of any additional oxalic acid formed by the fungus. It was pointed out that if excess oxalic acid be supplied to the fungus it should fall steeply to the normal equilibrium concentration, whereas if oxalic acid be supplied at a lower concentration there should be an increase to the normal level. The present work provides confirmation of this view.

Group N. The cultures of this group were grown under standard conditions for 14 days, then starved for 2 days on water. On the 16th day of growth *Cultures N10, 2, 3, 9, and 12* received 250 ml. of approximately 10 per cent. glucose, 10 per cent. glucose + 1 per cent. oxalic acid, 5 per cent. glucose + 1 per cent. oxalic acid, 1 per cent. oxalic acid, and 3 per cent. oxalic acid respectively.

Some of the data obtained are set out in Fig. 4.

It will be seen that the acid curves of N₂ and N₃ fall sharply until attaining the equilibrium level, when only a gradual fall is recorded. By contrast, in culture N₉, which received oxalic acid only, the acid concentration falls continuously since there is no glucose to maintain the equilibrium level.

The effect of oxalic acid in reducing its own formation from glucose was expected, but it is also found that added oxalic acid produces an effect on the CO_2 output. From Fig. 4 it will be seen that the CO_2 output of the culture N2 (—) supplied with 10 per cent. glucose + 1 per cent. oxalic acid is considerably in excess of that of the culture N10 (---) supplied with 10 per cent. glucose only.

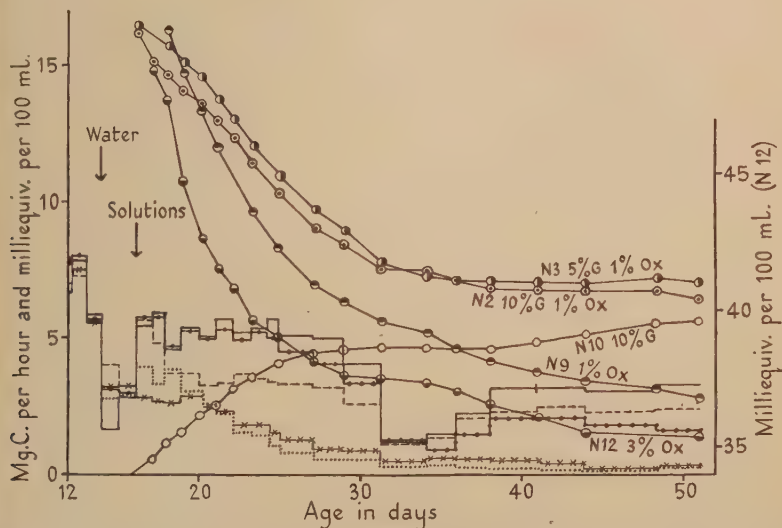


FIG. 4. CO_2 output and acid formation and utilization in Group N. CO_2 output is shown by the stepped graphs: N2 —, N3 —●—, N9 ×××, N10 ---, N12 Titratable acid is represented by the curves N2 —○—, N3 —●—, N9 —○—, N10 —○—, N12 —●—. On the 16th day the cultures received: N2, 10% glucose + 1% oxalic acid; N3, 5% glucose + 1% oxalic acid; N9, 1% oxalic acid; N10, 10% glucose; and N12, 3% oxalic acid.

Of the cultures supplied with oxalic acid only, the CO_2 output of N9 (×), receiving 1 per cent. oxalic acid only, is less than in any of the cultures supplied with glucose and there is no increase above the starvation rate. It is striking that the rate of CO_2 output is closely parallel with although somewhat higher than the rate of oxalic acid loss. In culture N12, which received 3 per cent. oxalic acid, for the first 4 days the rate of oxalic acid loss is in excess of that in culture N9 and the CO_2 output shows a corresponding increase above the starvation level. As in N9 the acid loss soon diminishes and the CO_2 output shows a similar reduction.

Group R. From the results of Group N it was concluded that the lower yields of oxalic acid on concentrated glucose solutions might be a result of the more rapid utilization of the acid. In the present group of cultures the effect of glucose concentrations on the rate of oxalic acid disappearance was studied.

Cultures R6, 7, 8, 9, 11, and 12 were grown under standard conditions, starved on tap-water for 2 days, and on the 19th day of growth supplied with

250 ml. of approximately 9 per cent. glucose+3 per cent. oxalic acid, 5 per cent. glucose only, 2 per cent. glucose+1 per cent. oxalic acid, 5 per cent. glucose+1 per cent. oxalic acid, 10 per cent. glucose+1 per cent. oxalic acid, and 17 per cent. glucose+1 per cent. oxalic acid, respectively.

The titratable acid values are set out in Fig. 5 and the rates of CO₂ output in Fig. 6.

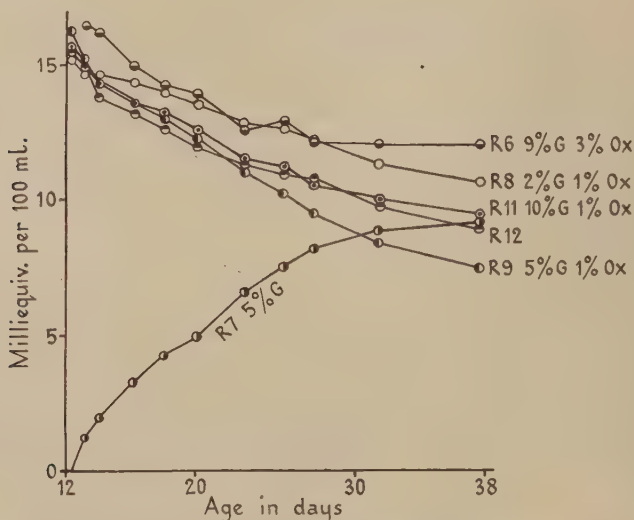


FIG. 5. Acid formation and utilization in Group R. Titratable acid is represented by the curves R6 —●—, R7 —○—, R8 —○—, R9 —○—, R11 —○—, R12 —○—. The cultures were supplied with 9% glucose+3% oxalic acid, 5% glucose only, 2% glucose+1% oxalic acid, 5% glucose+1% oxalic acid, 10% glucose+1% oxalic acid, and 17% glucose+1% oxalic acid respectively.

The results show that in the cultures of this group there is no constant increase in the rate of oxalic acid loss with increasing glucose concentration. The lowest rate of acid loss, however, is seen in R6, which was supplied with 9 per cent. glucose+3 per cent. oxalic acid.

No exact comparison of oxalic acid loss and CO₂ output can be made since it is possible that the formation of oxalic acid from glucose is proceeding continuously, although one would expect the presence of high concentrations of oxalic acid to retard its own formation. It may be significant, however, that the rates of CO₂ output are in the order to be expected from the rate of oxalic acid loss.

As would be expected from the results of Group N, the presence of oxalic acid causes an additional output of CO₂. All the cultures supplied with glucose+oxalic acid show an increase in CO₂ output above the starvation level, but this was not observed in culture R7 supplied with 5 per cent. glucose only. Three per cent. oxalic acid has evidently an inhibitory effect

on CO_2 formation since the CO_2 output of culture R6 with 9 per cent. glucose+3 per cent. oxalic acid was only a fraction of the CO_2 output of culture R11 with nearly the same concentration of glucose but with only 1 per cent. oxalic acid. The rate of glucose utilization was also less at the higher oxalic acid concentration. It should be pointed out, however, that in all cultures the rate of acid loss would account only for a small part of the CO_2 output.

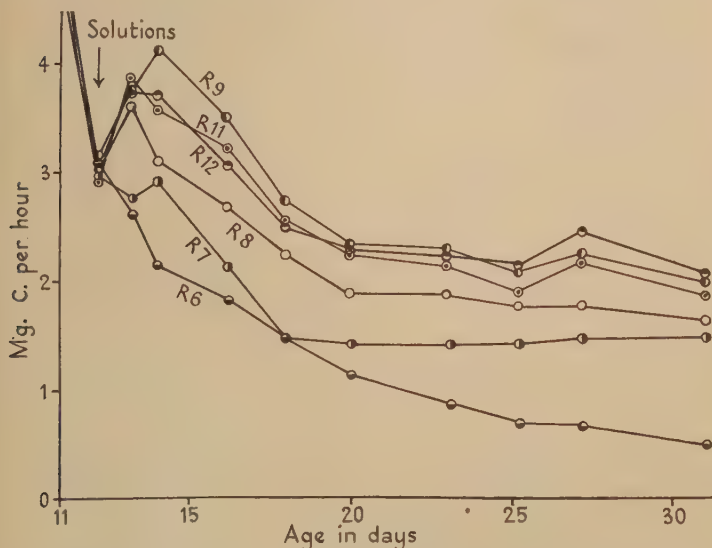


FIG. 6. CO_2 output of cultures R6—●—, R7—●—, R8—○—, R9—●—, R11—○—, R12—●—. On the 12th day of growth the cultures were supplied with 9% glucose+3% oxalic acid, 5% glucose only, 2% glucose+1% oxalic acid, 5% glucose+1% oxalic acid, 10% glucose+1% oxalic acid, and 17% glucose+1% oxalic acid respectively.

The results of this group indicate that there is a connexion between oxalic acid loss and CO_2 output, although it was not possible in this case to demonstrate an increase in the rate of oxalic acid loss with increase in glucose concentration.

Group S. This group of cultures supplies confirmation of the view that oxalic acid plays an important role in the formation of CO_2 .

It is usually held that neutralized oxalic acid is incapable of utilization by *A. niger*. This consideration leads to the expectation that a culture receiving glucose+neutral sodium oxalate will have the same CO_2 output as a culture supplied with glucose only. A culture with glucose+oxalic acid would be expected to have the highest CO_2 output, while cultures supplied with neutral sodium oxalate only should have a lower rate of CO_2 production than cultures receiving an equivalent amount of oxalic acid.

Cultures S2, 9, 10, 11, and 12 were grown under the standard conditions,

starved on distilled water for 2 days, and on the 9th day of growth supplied with 250 c.c. of approximately 5 per cent. glucose, 5 per cent. glucose + 1 per cent. oxalic acid, neutral sodium oxalate $\equiv$ 1 per cent. oxalic acid, 5 per cent. glucose + neutral sodium oxalate $\equiv$ 1 per cent. oxalic acid, and 1 per cent. oxalic acid respectively.

The rates of CO_2 output are set out in Fig. 7. The results reveal that, as expected, the rates of CO_2 output of cultures S2 (5 per cent. glucose) and

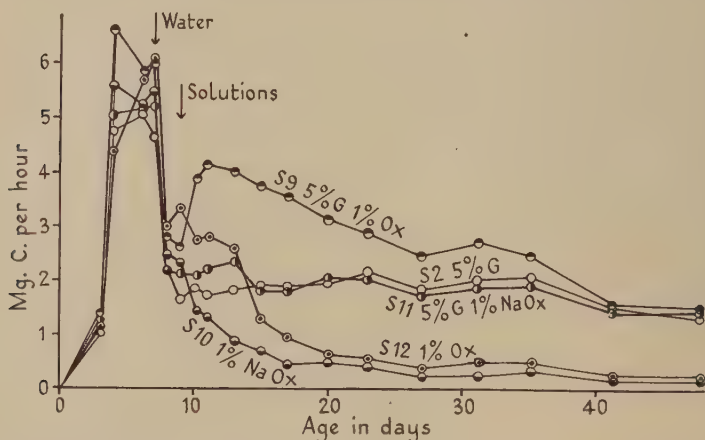


FIG. 7. CO_2 output of cultures S2 —○—, S9 —●—, S10 —◐—, S11 —◑—, and S12 —◔—. On the 9th day of growth the cultures were supplied with 5% glucose, 5% glucose + 1% oxalic acid, neutral sodium oxalate $\equiv$ 1% oxalic acid, 5% glucose + neutral sodium oxalate $\equiv$ 1% oxalic acid, and 1% oxalic acid respectively.

S11 (5 per cent. glucose + sodium oxalate) are almost identical. The CO_2 output of S10 supplied with oxalate only rapidly falls to a low level, while the respiration rate of culture S12 on oxalic acid only is higher than that of S10 but also diminishes to a low level. Culture S9 on glucose + oxalic acid has almost double the CO_2 output of S2 and 11. In culture S11 the neutral sodium oxalate does not retard the formation of oxalic acid which continues to accumulate until the acid salt is formed. In culture S10 supplied with sodium oxalate only there is a slight increase in the content of oxalate, evidently derived from the carbohydrate reserves of the mycelium.

The results of this experiment provide convincing evidence for the view that the disappearance of oxalic acid is associated with the formation of carbon dioxide.

Group T. In view of the strong support afforded by the results of Group S for the belief that oxalic acid plays an important role in CO_2 formation, an attempt was again made to influence the rate of oxalic acid disappearance by varying the concentration of glucose.

Cultures T1, 2, 3, 4, 5, and 6 were grown under standard conditions, starved on water for 2 days, and on the 13th day of growth received 250 ml.

of approximately 1 per cent. oxalic acid, 1 per cent. glucose+1 per cent. oxalic acid, 5 per cent. glucose+1 per cent. oxalic acid, 9 per cent. glucose+1 per cent. oxalic acid, 16 per cent. glucose+1 per cent. oxalic acid, and 5 per cent. glucose only respectively. The results are not published in detail since, as in Group R, there were no outstanding differences in the rates of acid loss or CO₂ output at the various glucose concentrations. The results of the present group of cultures, together with those of Group R, thus appear to show that the concentration of glucose has no effect on the rate of disappearance of oxalic acid supplied to the fungus, although the possibility is not excluded that any oxalic acid arising *within* the hyphae might be affected by the glucose concentration.

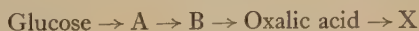
DISCUSSION

It has already been pointed out (Allsopp, 1937) that, in the utilization of glucose by *A. niger*, oxalic acid is produced and appears to attain an equilibrium concentration at which the rates of formation and decomposition of the acid are equal. In the present work an attempt was made to elucidate the nature of this supposed equilibrium.

In the three series of experiments (N, Qa, S) in which solutions of glucose were supplied to preformed mycelia, the maximum oxalic acid concentration and rate of acid formation diminished with increasing glucose concentration, while the CO₂ output increased with the concentration of glucose. Similar results were obtained in Group Qb in which the spores were sown on culture solutions containing the usual complement of mineral salts but with various concentrations of glucose substituted for the normal concentration of 5 per cent. In all cases in which glucose was present in excess a condition of equilibrium was attained. The equilibrium level varied gradually with the age of the culture, in some cases a slight increase but usually a gradual fall being recorded. Depletion of the glucose supplies was invariably followed by a steep fall to a low concentration of oxalic acid.

Other series of experiments (Groups N, R, S, and T) supplied information as to the effect of oxalic acid on the equilibrium level and rate of CO₂ production. In cultures supplied with glucose solutions containing oxalic acid at a concentration well above that arising in cultures supplied with glucose only, there was a steep fall in oxalic acid concentration to the normal equilibrium level. When oxalic acid only was supplied there was a continuous fall in concentration of the acid.

The results summarized above lead to the view that changes of the following type are proceeding:

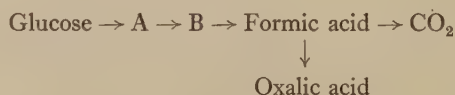


The accumulation of oxalic acid when glucose solutions are supplied to the fungus is a result of the rate of its formation exceeding that of its further conversion. Increase in the concentration of oxalic acid will retard its formation and accelerate its disappearance until finally the equilibrium level is

attained at which the two rates are equal. Addition of oxalic acid at a concentration above this level would be expected to retard its formation from glucose, but increase the rate of disappearance until the normal concentration is attained.

The observed effect of glucose in diminishing the equilibrium level also requires explanation. It appeared not unlikely that the rate of oxalic acid utilization might be increased by increasing concentrations of glucose, and two groups of experiments (R and T) were carried out to test this hypothesis. From these experiments, however, it would seem that glucose concentration has no effect on the rate of oxalic acid utilization. One cannot exclude the possibility, however, that glucose promotes the further conversion of oxalic acid arising within the hyphae and that in the case of externally supplied oxalic acid some other factor is limiting. On this view the oxalic acid normally appearing in the culture solution is that escaping direct transformation into the compound X.

Alternatively, one might adopt the suggestion of Chrzęszcz and Zakomorny (1933) that oxalic acid does not lie on the direct line of sugar degradation. They suggested the following scheme:



In such a scheme the amount of oxalic acid accumulating is dependent on the concentration of formic acid, which in turn is controlled by the rate of its conversion into CO_2 . If glucose accelerated the conversion of formic acid to CO_2 the observed effect of glucose concentration on the oxalic acid concentration would be accounted for. Similarly, some other suitable intermediate could take the place of formic acid in this explanation of the low oxalic acid yields at high glucose concentrations.

From the present and other work there is no doubt that *A. niger* can utilize oxalic acid, but there has been no convincing demonstration of the resulting products, although it is widely accepted that the acid is converted into carbon dioxide.

Several workers (Franke *et al.*, 1937, 1943; Niekerk-Blom, 1946) have studied oxalic acid destroying enzymes in mosses and other green plants, but no information of this kind is available for the fungi.

The present investigation, however, has revealed a certain relationship between the amount of oxalic acid lost and the CO_2 output. Despite the large number of experiments on this question, both in this and in the previous work (Allsopp, 1937), in not a single instance was the rate of oxalic acid loss in excess of the CO_2 output. In cultures supplied with oxalic acid alone the rate of CO_2 production was closely parallel with, but somewhat above, the rate of oxalic acid carbon loss. Cultures supplied with glucose+oxalic acid had a greater CO_2 output than cultures supplied with glucose only (Groups N, R,

and T), but addition of neutral sodium oxalate, which is incapable of utilization by the fungus, caused no increase in the CO_2 output from glucose solutions. The above results certainly indicate that the utilization of supplied oxalic acid is associated with the formation of at least an equivalent amount of CO_2 , but do not necessarily imply that the oxalic acid itself is converted to CO_2 , nor that oxalic acid is connected with CO_2 output in cultures supplied with glucose only.

Support for this latter belief, however, is afforded by the experiments with pure glucose of varying concentrations. The increase in CO_2 output with increase in the concentration of glucose is accompanied by a lower equilibrium level of oxalic acid which would be readily explained by a more rapid utilization of the acid at higher glucose concentrations. A comparison of the relative rates of oxalic acid formation and CO_2 output in cultures of different groups provides further support for the view that oxalic acid plays an important role in the normal respiration of the fungus. It was found that cultures which accumulate oxalic acid to a high concentration have usually a relatively low rate of CO_2 output, thus suggesting that the lower rate of CO_2 formation is due to the slow rate of oxalic acid utilization.

The above considerations lead to the view that CO_2 formation in the respiration of glucose by *A. niger* is intimately associated with the utilization of oxalic acid. It does not follow, however, that the oxalic acid itself is oxidized to CO_2 . The same result would be obtained if oxalic acid participated in a coupled reaction in which liberation of CO_2 was associated with the reduction of the acid. In either case, a coupled reaction is the most likely explanation of the postulated increased rate of oxalic acid utilization in the presence of glucose.

Lynen and Lynen (1948) propose a similar hypothesis to account for the observation of the present author (Allsopp, 1937) that pyruvic and lactic acids inhibit the formation of oxalic acid from glucose. They suggest that pyruvic acid yields an 'active acetic acid' which unites with oxalic acid to form oxalacetic acid. It may well be that a reaction of this type is responsible for the effect of high glucose concentrations in reducing the concentration of oxalic acid.

In the absence of further information it is scarcely desirable to elaborate any more detailed scheme of oxalic acid formation. The present work emphasizes the danger of drawing conclusions as to the chemical mechanism of oxalic acid formation, from feeding experiments in which hypothetical intermediates are supplied to the living fungus. Considerable advances would probably result if observations on the living material were supplemented by studies using non-living enzyme preparations. In any event, it may be concluded from the present work that oxalic acid plays an important role in the respiration of *A. niger*.

SUMMARY

1. Some recent works on the formation of oxalic acid by various fungi are critically considered.

2. The present work deals with the role of oxalic acid in the metabolism of *Aspergillus niger*.

3. When glucose solutions were supplied to preformed mats of the fungus oxalic acid accumulated, attaining an equilibrium level which was not exceeded despite the presence of a considerable concentration of glucose.

4. When the glucose supplies were depleted the oxalic acid concentration fell steeply to a low level.

5. The concentration of oxalic acid was dependent on the glucose concentration. In three separate series of experiments it was shown that the oxalic acid concentration diminished with increasing glucose concentration.

6. Similar results were obtained when the cultures were reared from spores on culture solutions with the normal amounts of nutrient salts but different glucose concentrations.

7. In all cases the CO_2 output increased with the glucose concentration.

8. When cultures were supplied with glucose+oxalic acid, the concentration of the latter fell steeply to the equilibrium level attained on glucose only. In a culture receiving glucose+oxalic acid, with the oxalic acid concentration somewhat below the normal equilibrium concentration, the formation of oxalic acid from the glucose ceased as soon as the equilibrium level had been attained.

9. When 1 per cent. oxalic acid only was supplied to the fungus the concentration gradually diminished to a low level. When 3 per cent. oxalic acid was supplied the rate of acid utilization soon fell to a low value.

10. In several experiments it was shown that the rate of CO_2 output was higher from cultures supplied with glucose+excess oxalic acid than from cultures supplied with glucose only.

11. The rate of oxalic acid carbon loss was always below that of the CO_2 carbon output both in cultures supplied with oxalic acid only and in cultures receiving glucose+oxalic acid.

12. The cultures were incapable of utilizing neutral sodium oxalate and the presence of this substance had no effect on the CO_2 output.

13. The results indicate that the utilization of oxalic acid is associated with the liberation of at least an equivalent amount of CO_2 .

14. It is suggested that the utilization of oxalic acid is promoted by the presence of glucose, thus accounting for the lower oxalic acid concentrations and higher rates of CO_2 output of cultures with higher glucose concentrations.

The present work was begun in the Department of Botany, University of Manchester, at the suggestion of Professor T. A. Bennet-Clark, to whom I am indebted for helpful advice. I also wish to thank Professor C. W. Wardlaw for his interest and friendly criticism.

LITERATURE CITED

- ALLSOPP, A. (1937). The formation of oxalic acid by *Aspergillus niger*. *New Phytol.* **36**, 327.
- BENNET-CLARK, T. A. (1933). The role of the organic acids in plant metabolism. Part III. *Ibid.* **32**, 197.
- and LA TOUCHE, C. J. (1935). The utilization of organic acids by *Aspergillus niger*. *Ibid.* **34**, 211.
- CHRZĄSZCZ, T., and ZAKOMORNY, M. (1933). Biochemische Umbildung der Ameisensäure durch Schimmelpilze und Chemismus dieses Vorganges. *Biochem. Z.* **263**, 105.
- FRANKE, W., and HASSE, K. (1937). Zur biologischen Oxydation der Oxalsäure I. *Z. physiol. chem.* **249**, 231.
- SCHUMANN, F., and BANERJEE, B. (1943). Zur biologischen Oxydation der Oxalsäure II. *Ibid.* **278**, 24.
- HAMADA, M. (1940). Physiologisch-morphologische Studien über *Armillaria mellea* (Vahl) Quéél., mit besonderer Rücksicht auf die Oxalsäurebildung. Ein Nachtrag zur Mykorrhiza von *Galeola septentrionalis* Reichb. *Jap. J. Bot.* **10**, 15.
- JAQUOT, R. (1938a). La Fermentation oxalique. Mécanisme de l'oxalicogénèse chez les mucédinées. *Ann. des Fermentations*, **4**, 284, 346.
- (1938b). La production d'acide oxalique par diverses moisissures. Influence du pH. *Compt. Rend. Soc. Biol.* **127**, 1431.
- (1938c). La production d'acide oxalique par diverses moisissures. Oxalicogénèse et dépenses énergétiques. *Ibid.* **128**, 69.
- LEIBOWITZ, J., and HESTRIN, S. (1945). Alcoholic fermentation of the oligosaccharides. *Advances in Enzymology*, **5**, 87.
- LYNEN, F., and LYNEN, F. (1948). Zum biologischen Abbau der Essigsäure IV. Die Bildung von Oxalsäure durch *Aspergillus niger*. *Ann.* **560**, 149.
- NIEKERK-BLOM, C. J. (1946). Notes on the oxidation of oxalic acid by mosses. *Proc. Koninkl. Nederland. Akad. Wetenschap.* **49**, 1096.
- NORD, F. F., and MULL, R. P. (1945). Recent progress in the biochemistry of *Fusaria*. *Adv. Enzymology*, **5**, 165.
- and VITUCCI, J. C. (1947). On the mechanism of enzyme action, XXIX. The acetate metabolism of certain wood-destroying molds and the mechanism of wood decay. *Arch. Biochem.* **14**, 229.
- (1948). Certain aspects of the microbiological degradation of cellulose. *Adv. Enzymology*, **8**, 253.
- PERLMANN, D. (1948). On the nutrition of *Sclerotium Delphinii*. *Amer. J. Bot.* **35**, 360.
- SMITH, VIRGINIA M. (1949). On the mechanism of enzyme action, XXXVI. Dehydrogenation studies on *Merulius lacrymans* and *Marasmius chordalis*, and the mechanism of oxalic acid formation. *Arch. Biochem.* **22**, 275.

The Effects of Sugar and Potassium on Extension Growth in the Root

R. BROWN AND J. F. SUTCLIFFE

Botany Department, University of Leeds

INTRODUCTION .

THE purpose of this investigation is to analyse the nature of the promoting effect of certain nutrients on extension growth in the root, in circumstances in which extension is not affected by the activity of the meristem or of the mature tissues in the body of the root. Such influences may of course arise when the experimental material is intact roots growing in pure culture or attached to a parent plant. The technique that is used here involves observations on the growth of short fragments excised from the extending zone of the root. It has been shown that these fragments continue to grow when they are supplied with suitable media and that the growth is due to cell extension only. This material, however, not only provides a simpler experimental situation than the intact root, but it also yields data which may be compared directly with those obtained with coleoptile segments. Extensive observations have been made by various workers on the conditions which promote extension in the coleoptile by the use of the technique elaborated by Bonner (1934) which is based on excising 3-mm. fragments from the body of a coleoptile. The tissue used here is immediately comparable with the coleoptile fragments since like these it consists only of extending cells. It may be noted that the relative increase in length that occurs in these root fragments may be considerably greater than the increases that have been recorded with the corresponding coleoptile material, and they may therefore be the more suitable objects for the study of the general characteristics of extension.

The results presented below are part of a more extensive series which were obtained with segments taken from roots of two species, *Zea* and *Cucurbita*. The bulk of the results from *Cucurbita* have been omitted, however, since although they resemble closely those obtained with *Zea* they may be the less reliable. The growth of the *Cucurbita* tissue may involve the production of lateral roots which are never produced by the material from *Zea*.

In a preliminary series of experiments with *Cucurbita* the effects of a large number of substances on extension growth were examined, but only three, sugar, potassium, and calcium salts, were found to have promoting effects. The following substances either had no effect or a depressing one: alanine, glycine, asparagine; malic, fumaric, and succinic acids; sulphates, nitrates, and phosphates; magnesium and ferric ions; yeast extract, ascorbic acid, vitamin B₁, adenylic acid, and heteroauxin. With maize the sugars and potassium again gave marked stimulation, but this tissue is apparently indifferent to calcium. Of the sugars with maize the most vigorous growth was given with

sucrose, glucose being slightly less effective than sucrose, and fructose less than glucose.

The preliminary series of experiments further showed that potassium is effective only in the presence of sugar. Clearly the results indicated that the primary requirement for growth is a supply of carbohydrate, and accordingly a series of observations was made to determine the relation between growth and the absorption and utilization of sugar both in the presence and in the absence of potassium. The utilization of sugar has been analysed in terms of changes in dry weight, cellulose content, respiration, and free soluble sugar content. Since the utilization of sugar is likely to be affected by the partial pressure of oxygen, the effect of a low partial pressure in these several connexions has also been examined.

It may be noted that the experimental material used here resembles the coleoptile in responding to potassium and sugar (Schneider, 1938; Thimann and Schneider, 1938), but differs from it in being indifferent to malic, succinic, and fumaric acids (Thimann, 1948), and in being markedly depressed in growth by concentrations of heteroauxin that normally stimulate the coleoptile. In certain instances slight stimulations at very low dilutions of heteroauxin were recorded. These stimulations, however, are very much smaller than the stimulations given with the coleoptile. With intact roots Burström (1942) found that root cells were stimulated in the early stages of extension. Such an effect was not observed in these experiments, probably because all the cells of the segments had already traversed the stage when they could respond to heteroauxin at the time of excision. The failure of the segments to respond to nitrates and sulphates also requires some comment, since Burström (1947) using intact roots found that nitrates stimulate extension, and Brown and Rickless (1949) using fragments that included the apical meristem that sulphates as well as nitrates promote extension. In the experiments of Burström and of Brown and Rickless, however, the experimental conditions were applied throughout the development of the cell, whereas in the experiments of the present series they were only applied after the cells had probably accumulated a reserve of inorganic nutrients in the vacuole, and the failure to respond to certain substances in the medium may have been due to the same nutrients being already available.

MATERIALS AND METHODS

Experimental material. The segments are obtained from seeds germinated on damp filter-paper for 48 hours at 25° C. in the dark. Consistent results are only obtained when the seeds are germinated with constant moisture conditions, and only when roots of approximately the same length are used as parent material. In this investigation only roots 2.0–3.0 cm. in length have been used.

The segment that is excised occupies the zone in the parent root 1.5–3.0 mm. from the tip. The choice of this material is based on the results of a series of experiments which were designed to show how the capacity for growth varied

with the size of the fragment and with distance from the apex of the root. The data of Fig. 1 show the lengths reached after 18 hours by the second and successive segments each 1.5 mm. in length cut from the apex towards the base of the root and cultured with 2 per cent. sucrose. Clearly the first segment of the series grows markedly more vigorously than the rest. It consists almost entirely of vacuolated cells and it has been shown by three techniques

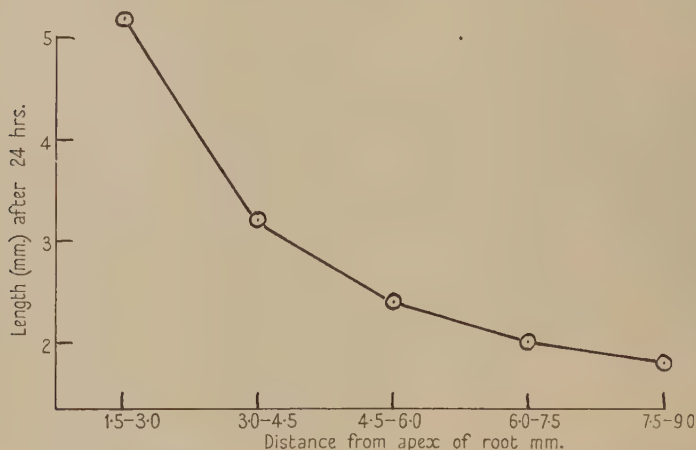


FIG. 1. Lengths of segments excised from different zones of the root after culturing for 24 hours in 2 per cent. sucrose. All segments originally 1.5 mm. in length and distance of front and rear edges of original segments from apex of root indicated by figures along the abscissa.

that the increase in length that occurs in it is due only to extension. The tissue has been examined for the occurrence of mitotic figures at different stages of growth by applying the aceto-carmin squash technique, and no mitotic figures have been found at any stage of growth. Secondly, the number of cells in successive rows of the cortex before and after extension have been determined, and no change has been recorded. The counts in this connexion have been made on tissue that has been flattened in the aceto-carmin squash procedure, since it has been found with this technique that cells tend to separate along longitudinal but not transverse walls, and ranks of cells thus tend to remain intact. Thirdly, determinations of the total number of cells in the segments before and after extension have been made by the technique described by Brown and Rickless (1949), and it has been found that there is no increase during growth.

At the time of excision the segment consists almost entirely of cells that are in an early stage of extension. It contains at this stage provascular strands, and a general cortical zone can be distinguished from a central stelar region. During growth the cells of the provascular strand increase in length and some of them differentiate into xylem elements with spiral thickenings. Root hairs

are not present when the segment is excised, but they may develop towards the end of the experimental period. In the segments of *Zea*, lateral roots are not developed and a secondary meristem does not form over either of the cut surfaces.

The possibility has been considered that the development of xylem elements may restrict the growth of the segment as a whole. This possibility may be disregarded, however, since lignification only occurs towards the end of the experiment, and segments which are cut longitudinally do not bend either towards or away from the wounded surface.

Each experiment may require hundreds of segments and a knife has therefore been devised for excising them rapidly and accurately. This consists in principle of two safety-razor blades clamped in a holder 1.5 mm. apart and these are pushed into a channel which is just slightly more than 1.5 mm. wide. The roots are stretched across the channel, each traversing a perforation in the support on one side and having the apex accommodated in a pit 1.5 mm. deep in the support on the opposite side.

General experimental design. Segments have been cultured in 5.0 per cent. sucrose, 2.0 per cent. sucrose, 0.25 per cent. sucrose, and water. The 2.0 per cent. sucrose medium has been used with an atmosphere containing 20 per cent. oxygen (air) and one containing 5 per cent. oxygen. Each nutrient medium has been used with and without potassium chloride, which when applied has been dissolved in the medium at the rate of 300 mg. per litre, this having been found to be the optimum concentration for growth. Thus the investigation involves observations with ten treatments.

With each treatment the following observations have been made: increase in length, absorption of sugar, cellulose content, dry weight, internal free sugar, and respiration.

Each set of observations has been made with serial samples taken usually at intervals of 12 hours over an experimental period of 48 hours. The segments are cultured in batches of twenty in flasks, and at the beginning of the experiment, when the sample size is two flasks, eight flasks are set up for a single set of observations. If the sample size is three flasks, then twelve are set up. On each sampling occasion the appropriate number of flasks are withdrawn and the relevant observations made on the segments they contain.

Usually each set of observations has been made with a different independent experiment. Only the cellulose and dry weight measurements have been made on the same material. The potassium effect has usually been investigated with two experiments set up at the same time, one with potassium in the medium and the other without it.

Cultural technique. The segments together with the culture fluid are accommodated in 25-c.c. flasks with wide necks which are shaken in a constant-temperature bath maintained at 25° C. Twenty segments with 2 c.c. of culture fluid are placed in each flask. The relevant data given below indicate the importance of adequate aeration for vigorous growth, which in this technique is provided by continuous shaking. The possibility that growth may be

depressed by simple mechanical agitation has been examined by comparing growth after 24 hours with segments that have been shaken and with others that have been cultured on sintered-glass disks. There is little if any difference between the results obtained with the two methods. If anything, the segments that have been shaken attain the greater length.

Each culture flask is closed by a rubber stopper which is traversed by two thick-walled capillary tubes each 9 in. long, the lower ends of which only reach as far as the lower surface of the stopper. The upper ends of the two tubes are held firmly in clamps attached to a horizontal wooden platform. The platform is arranged above the surface of the water in a constant-temperature bath, and when in position the flasks hang from the platform but immersed in the water of the bath. Ten flasks may be attached to the platform, which by means of a suitable mechanical arrangement is caused to move in a circular path in its own plane, thus carrying the flasks through the water in the same path.

In experiments in which the fluid is required to be in equilibrium with air, the upper ends of the two tubes from each flask are open to the atmosphere. During shaking with this arrangement there is little or no reduction in the volume of fluid in each flask since the narrow bore of the capillary tube reduces loss through evaporation. In some experiments a low partial pressure of oxygen above the fluid is required, and this is secured by circulating an appropriate gas mixture through the flasks for 30 minutes while they are being shaken, and then closing the upper ends of the tubes with short lengths of pressure tubing provided with screw clips. With this arrangement there is a possibility that growth may be influenced by the accumulation of carbon dioxide in the flask. It has been found, however, that closing the tubes when air is in the flasks has no effect on growth, and this possibility has therefore been disregarded.

The flasks are covered with a dense black paint to exclude light. The effect of light has not been examined in detail, but it was found that the intermittent light from a lamp that formed the heating element in the constant-temperature bath was sufficient to reduce growth by about 10 per cent.

Sterile conditions are not maintained in the cultures, and frequently when sugar has been provided in the medium towards the end of the experiment the solutions are faintly turbid. Bacterial contamination, however, does not apparently affect growth, since although the degree of such contamination varies considerably, a corresponding variation in growth has not been observed.

Growth measurements. In the normal procedure, growth is measured as change in length with time. The lengths at different times are determined with different samples, since the manipulations incidental to making the measurements depress subsequent growth.

Two flasks are taken for each sample. The twenty segments they contain are mounted on microscope slides, and the length and breadth of each segment is measured microscopically against a calibrated micrometer eyepiece using a 2-in. objective. A mean is calculated from the forty observations, and each

value for growth given in the next section is such a mean. During growth there is little or no change in breadth, and although the measurements of breadth were made as a standard procedure they are not given here.

The general reliability of the cultural and measurement techniques with respect to growth may be assessed from the results of four replicate experiments with 0.25 per cent. sucrose given in Table I, from which it is evident that duplicate determinations rarely differ by more than about 5 per cent.

TABLE I

Changes in length (mm./segment) with time in four replicate experiments with 0.25% sucrose

Hours	I	II	III	IV
4	2.32	2.42	2.14	—
6	—	—	—	2.42
8	2.82	2.73	2.79	—
12	3.39	3.55	3.41	3.42
24	4.06	3.88	4.08	4.14
36	—	4.03	4.27	4.27

Determination of dry weight. The dry weight is measured by weighing a known number of segments (usually 60) after heating in a hot-air oven at 85° C. for 3 hours. More prolonged heating does not affect the result, and the technique used gives values which are almost identical with others obtained with freeze drying.

Determination of the cellulose content. The cellulose content has been estimated by boiling the dry tissue with 3 per cent. sulphuric acid for 3 hours under a reflux condenser. After treatment the segments are washed, dried at 85° C., and weighed. This technique is based on one due to Schulze and described by Czapek (1922), who recommends that the tissue should be ground before treatment with sulphuric acid. Since, however, only gross effects are being investigated, the grinding procedure has been omitted. The acid treatment removes all cytoplasmic components, starch, hemicelluloses, and mucilages, but it is doubtful whether the residue is cellulose only. At the same time, it is probably predominantly cellulose and similar structural components of the wall. When we use the term cellulose content below we do so with the qualifications required by the imperfections of the technique.

Determination of respiration rate. The change in the rate of oxygen absorption with time has been measured by setting-up a cultural series in the normal way, at intervals taking samples of two flasks, and measuring over a period of 2 hours the respiration of the sample in a Barcroft respirometer. The segments are transferred to the respirometer flasks together with the fluid in which they have been cultured. This provision, while it facilitates relating respiration data to the growth data, introduces the complication that the oxygen uptake that is measured may be due partly to bacteria in the medium. This has been allowed for, however, by determining the oxygen uptake of the medium after removal of the segments and deducting this from the uptake measured when the segments are present.

Measurement of sugar absorption. The absorption of sugar is measured by taking a sample at intervals from a cultural series, determining the sugar content of the fluid of each flask, and comparing this with the known sugar content at the beginning of the experiment.

After diluting the culture fluid when necessary, the hexose content is determined immediately on an aliquot, and the sucrose content by measuring the increase in reducing power after hydrolysis with invertase. The reducing power is determined by the Hagedorn-Jensen technique (Streptkov, 1937). The titration values given before hydrolysis are expressed in the next section as milligrams of invert sugar.

The estimation of reducing sugar in the medium is necessary since it has been found that sucrose is hydrolysed by the tissue and hexose accumulates in the medium.

Clearly the sugar that is absorbed may promote an increase in dry weight or it may be consumed in respiration. From the present series of results approximate estimates can be made of the amount that is involved in each connexion and the total of the two estimates for any period may be compared with the directly determined absorption for the same period. A series of such comparisons is provided by the data of Table II.

TABLE II

Absorption of sugar after different times from 0.25, 2.0, and 5.0% sucrose solutions calculated (C) and directly determined (D) (mg./20 segments)

Hrs.	0.25		2.0		5.0	
	D	C	D	C	D	C
12	1.7	1.1	3.8	2.6	7.6	3.8
24	3.2	2.6	6.6	6.6	12.9	8.3
36	4.0	3.3	11.1	9.7	18.6	13.8
48	4.4	4.1	15.2	13.3	24.0	18.7

It is evident that the differences between the determined and calculated values may be large, although it may be noted that the largest differences which are given with the 5 per cent. medium only represent 6 per cent. of the total amount of sugar originally present in the culture fluid. The differences, however, are probably due to (a) sampling errors of three separate experimental series; (b) errors due to bacterial contamination which affect only the directly determined values; (c) evidence is available which suggests that substances are produced from the products of hydrolysis which have a low or negligible reducing power. (b) and (c) are likely to exaggerate the directly determined values and this corresponds with the data of Table II, in which the calculated values are usually smaller than the directly determined.

At the same time the directly determined absorption values do indicate large gross effects, and the data on which they are based are of particular relevance in evaluating the effect of changes in the external medium on changes in growth.

Internal sugar content. This has been determined by extracting the sample with hot 95 per cent. alcohol for 3 hours, evaporating the solution almost to dryness under reduced pressure at 40° C., and determining the reducing and sucrose fractions in the residue after this has been redissolved in a known volume (Strepkov, 1937). The sucrose fraction has been estimated from the increase in reducing power after hydrolysis with invertase.

The sample after it is withdrawn from the flask is washed in several changes of water before it is extracted. It is doubtful, however, if this treatment removes all the sugar that is not in the protoplast or the vacuole, since although the sucrose content increases with the increasing concentration of sugar, it is not affected by a decrease in the partial pressure of oxygen which markedly reduces absorption. This suggests that some sugar may be adsorbed on to the cell walls and some may be held in the intercellular spaces. This source of error, however, does not affect the comparative values of the data, since in some series at least 80 per cent. of the total amount of sugar in the tissue is reducing sugar.

In the next section, values are given for the total sugar content of the medium and the tissues in milligrams. These have been calculated from the titration figures with the assumption that the reducing fraction consists only of hexose.

EXPERIMENTAL RESULTS

Presentation of results. When the observations have been made with air in the flasks, each group of determinations is presented separately. Thus the observations on growth, respiration, absorption of sugar, internal sugar content, dry weight, and cellulose content are each presented separately when the experimental conditions involve a high partial pressure of oxygen. In each case the data are accompanied by the results of the relevant statistical analysis, except with the sugar-absorption data for which no analysis has been made, since as indicated above the errors in this determination are large. Each of these groups is also presented in graphical form in a figure which incorporates the corresponding data obtained with a low partial pressure of oxygen.

The several sets of results from experimental series in which the tissue is exposed to 5 per cent. oxygen are assembled in a separate and final table.

Growth. The effects of the several media on increase in length when the segments are exposed to air are shown by the data of Table III.

The data of Table III are reproduced in Fig. 2. The concentration of sugar evidently has a profound effect on extension. It may be noticed that the interaction term between time and sugar concentration is highly significant. This suggests significant differences between the times during which extension proceeds in the several media, and the data indicate that the time increases as the sugar concentration is increased from 0 to 5.0 per cent. sucrose. In water, extension is complete in 12 hours, in 0.25 per cent. sucrose in 24 hours, in 2.0 per cent. sucrose at 36 hours, and in 5.0 per cent. sucrose probably at 48 hours. These differences in time of extension contribute to establishing different final lengths in the different series. Final length increases as the

TABLE III

Change in length in water (0), 0.25, 2.0, and 5.0% sucrose, in the absence (—K) and in the presence (+K) of potassium. Lengths in mm.

Hrs.	0		0.25		2.0		5.0	
	—K	+K	—K	+K	—K	+K	—K	+K
4	2.14	2.10	2.14	2.26	2.21	2.25	2.15	2.13
8	2.23	2.20	2.79	3.04	3.03	3.15	2.74	2.79
12	2.21	2.25	3.41	3.76	3.75	3.95	3.03	3.36
24	2.27	2.28	4.08	5.02	5.21	5.73	4.89	5.17
36	2.27	2.22	4.27	5.19	5.75	6.45	5.71	6.04
48	2.28	2.25	4.19	5.21	5.78	6.48	5.89	6.28

Results of statistical analysis of above data. C indicates sugar concentration, K potassium, and T time. HS indicates significant at 1% level and S at 5%

Variance due to:	Degrees of freedom	Mean square	V.R.	
Time	5	9.8323	561.8	HS
Sugar	3	12.0611	689.2	HS
Potassium	1	1.0621	60.7	HS
C × K	3	0.2013	11.5	HS
T × C	15	1.2182	69.6	HS
K × T	5	0.0878	5.0	HS
Error	15	0.0175	—	—

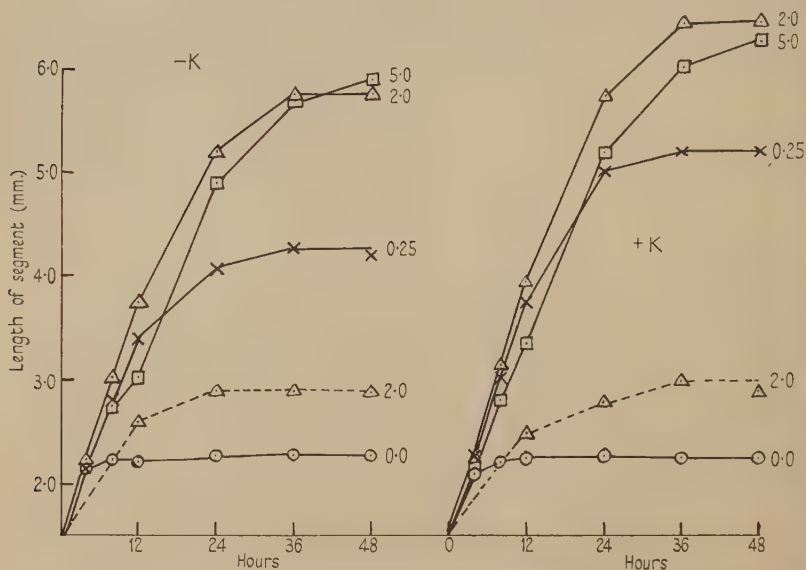


FIG. 2. Change in length with time with different treatments. Figures with the curves indicate percentage concentration of sucrose (0.0 indicates water). Continuous lines indicate air and broken lines 5 per cent. O_2 in atmosphere. +K and —K indicate presence and absence of KCl in the medium.

concentration is raised from 0 to 2.0 per cent. The final length, however, is also affected by the rate. The curves of Fig. 2 show that in each 12-hour interval during which extension is proceeding, the rate increases with increasing concentration of sugar from 0 to 2.0 per cent. During the first 12-hour interval the rate with 5.0 per cent. sugar is lower than it is with 2.0 per cent., but thereafter the slopes of the curves are steeper with the 5.0 per cent. medium. The length at 48 hours with the 5.0 per cent. medium is about the same as it is with the 2.0 per cent. medium, since although the rate after 12 hours is higher with the more concentrated medium, the rate before this time is lower.

The potassium effect is highly significant, and the data show that this ion stimulates growth. The interaction term between potassium and sugar concentration is also highly significant, and the data indicate that this is referable to the potassium effect being restricted to series in which sugar also is provided. With water alone potassium has no effect whatever. The interaction term between potassium and time is just significant at the 1 per cent. level. The data show that there is little or no effect on the time of extension, and the greater final lengths with potassium are primarily due to effects on rate.

Dry weight increase. The effect of nutrient treatment on dry weight increase when the cultures are exposed to air is shown by the data of Table IV.

TABLE IV

*Effect of nutrient treatment on dry weight (mg./20 segments).
Symbols as in Table III*

Hrs.	0		0.25		2.0		5.0	
	-K	+K	-K	+K	-K	+K	-K	+K
12	3.84	3.72	4.69	4.64	6.04	6.22	7.17	7.35
24	3.51	3.59	5.32	5.01	9.02	9.10	10.64	10.42
36	3.22	3.19	5.41	5.47	11.02	12.18	15.07	14.48
48	3.06	3.21	5.56	5.68	13.61	13.96	18.85	18.94

Statistical analysis

Variance due to:	D.F.	Mean square	V.R.	
Time	3	35.6896	603.9	HS
Sugar	3	152.0082	2,572.1	HS
Potassium	1	0.0435	—	—
C × T	9	12.6255	213.6	HS
C × K	3	0.1302	2.2	—
K × T	3	0.0318	—	—
Error	9	0.0591	—	—

The data of Table IV are reproduced in Fig. 3. The sugar effect is highly significant and it is clear that dry weight increases with increasing concentration of sugar. Dry weight increases with time when sugar is supplied, but decreases when water only is provided, and this corresponds to the significant interaction between time and sugar concentration. Change in dry weight with time is not affected by the presence of potassium.

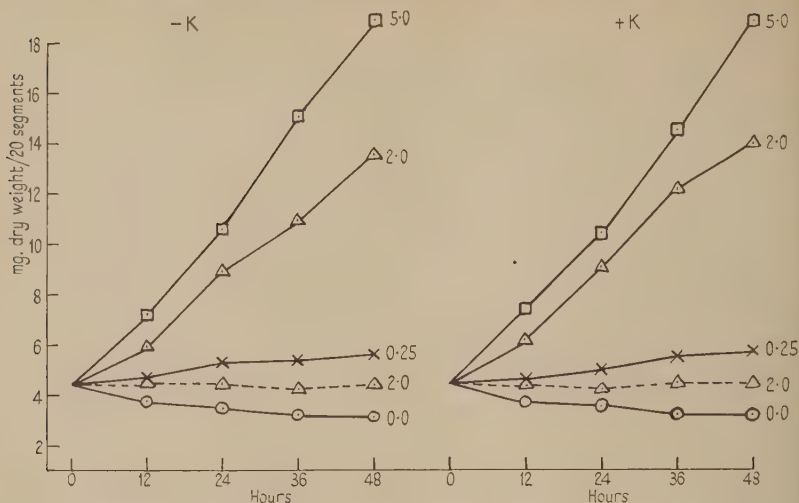


FIG. 3. Change in dry weight with time with different treatments. Symbols as in Fig. 2.

Cellulose content. The effect of nutrient treatment on cellulose content when the cultures are exposed to air is shown by the data of Table V.

TABLE V

*Effect of nutrient treatment on cellulose content (mg./20 segments).
Symbols as in Table III*

Hrs.	0		0.25		2.0		5.0	
	-K	+K	-K	+K	-K	+K	-K	+K
12	0.78	0.81	1.16	1.18	1.40	1.56	1.54	1.35
24	0.62	0.54	1.28	1.27	2.32	2.44	2.18	2.06
36	0.51	0.53	1.56	1.45	3.28	3.65	3.32	3.58
48	0.43	0.49	1.64	1.59	4.14	4.75	4.49	4.66

Statistical analysis

Variance due to:	D.F.	Mean square	V.R.	
Time	3	3.7907	364.5	HS
Sugar	3	10.8010	1,038.6	HS
Potassium	1	0.0504	4.9	—
K × C	3	0.0520	5.0	S
C × T	9	1.1862	114.1	HS
K × T	3	0.0224	—	—
Error	9	0.0104	—	—

The data of Table V are reproduced in Fig. 4. The effect of sugar concentration is again highly significant, and the data indicate that cellulose content increases with increasing concentration of sugar. The interaction term between time and sugar concentration is also highly significant, which corresponds to the position that cellulose content decreases with time when water only is supplied, but increases in all media containing sugar.

The overall potassium effect is not significant, but the interaction between potassium and sugar is significant at the 5 per cent. level. This may indicate that there is a slight stimulating effect of potassium with concentrated solutions of sugar, but no effect with water and with the 0.25 per cent. medium.

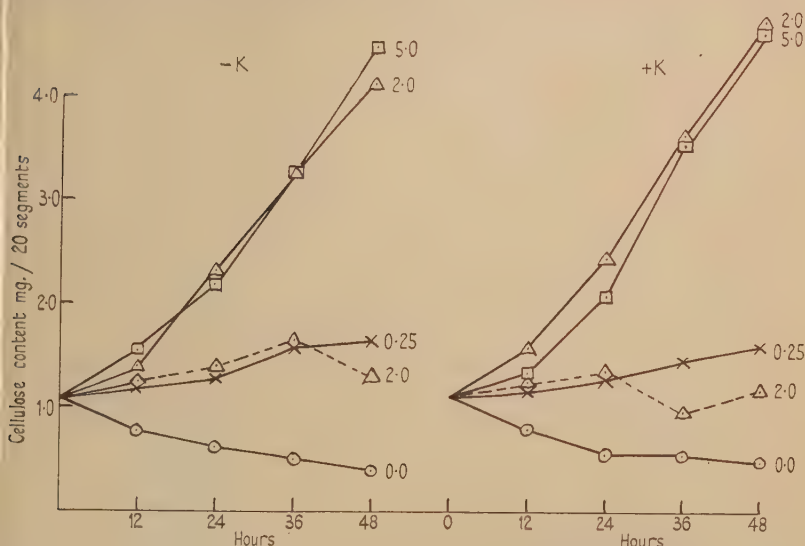


FIG. 4. Change in cellulose content with time with different treatments.
Symbols as in Fig. 2.

It may be noted in this connexion that Bonner (1934) also found that cellulose is formed when coleoptile segments are suspended in fructose solutions containing heteroauxin. The results reported by this worker, however, suggest that the root and the coleoptile may differ in this respect since although in the former hydrolysis of wall constituents apparently occurs when sugar is not supplied, in the coleoptile on the contrary in these conditions a slight synthesis of cellulose continues.

Absorption of sugar. The absorption of sugar after different times from the different media when the cultures are exposed to air is shown by the data of Table VI.

TABLE VI

*Amounts of sugar absorbed from different media (mg./20 segments).
Symbols as in Table III*

Hrs.	0.25		2.0		5.0	
	-K	+K	-K	+K	-K	+K
12	1.74	1.70	3.8	5.1	7.6	6.0
24	3.18	3.14	6.6	7.4	12.9	12.2
36	4.04	4.08	11.1	11.9	18.6	18.6
48	4.37	4.44	15.2	15.8	24.0	25.2

The data of Table VI are reproduced in Fig. 5. The data show that sugar absorption increases with increasing concentration in the medium. Moreover, they suggest that the rate of absorption when high levels of sugar are available remains more or less constant with time. The data do not indicate that there is an effect of potassium on absorption. On the other hand, as indicated above, the error involved in the determination of absorption in this investigation has

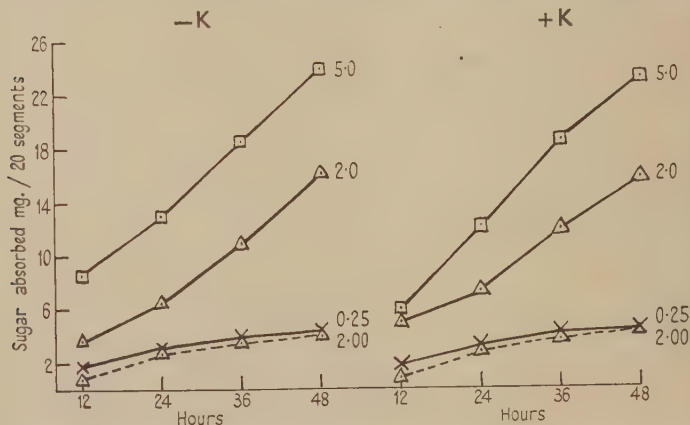


FIG. 5. Sugar absorbed from the medium after different times with different treatments. Symbols as in Fig. 2.

been high, and since potassium stimulates respiration and does not depress dry weight increase, an enhancement of absorption in the presence of potassium might occur. At the same time it may be emphasized that whether potassium has a slight effect on absorption or not, it certainly does not have a large and pronounced effect.

Free sugar content. The effect of nutrient treatment on the sugar content when the cultures are exposed to air is shown by the data of Table VII.

The data of Table VII are reproduced in Fig. 6. It is evident that the internal sugar content increases with increasing concentration in the medium. The data and the significant interaction between time and sugar concentration in the medium indicate that while the content decreases with time when water alone is provided, it increases with time in all sugar media. In this series of observations the potassium effect is significant, and the data indicate that potassium depresses the sugar content, although it probably has no effect in the absence of sugar.

Respiration. The effect of nutrient treatment on respiration when the cultures are exposed to air is shown by the data of Table VIII and Fig. 7.

The sugar effect is highly significant, and the data indicate that respiration increases with increasing concentration of sugar. The time and sugar interaction is highly significant. Clearly after 12 hours, whereas with 0 and 0.25 per cent. sugar respiration decreases steadily with time, with 2 per cent. it

TABLE VII

Effect of nutrient treatment on sugar content (mg./20 segments).
Symbols as in Table III

Hrs.	0		0.25		2.0		5.0	
	-K	+K	-K	+K	-K	+K	-K	+K
12	0.63	0.59	1.00	0.94	1.71	1.53	3.34	3.39
24	0.50	0.47	1.34	1.22	3.40	3.24	4.80	4.65
36	0.38	0.41	1.78	1.59	4.52	4.41	7.75	7.30
48	0.29	0.31	1.92	1.64	5.30	5.12	10.10	9.82

Statistical analysis

Variance due to:	D.F.	Mean square	V.R.	
Time	3	11.0177	1,551.8	HS
Sugar	3	55.8237	7,862.5	HS
Potassium	1	0.1405	19.8	HS
K × C	3	0.0160	2.3	—
C × T	9	3.8293	539.3	HS
K × T	3	0.0068	—	—
Error	9	0.0071	—	—

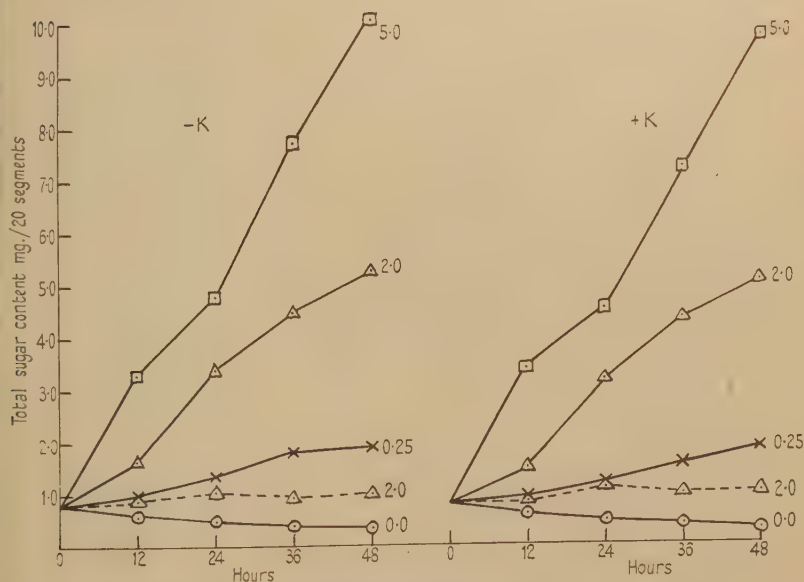


FIG. 6. Change in free sugar content of tissue with time with different treatments.
Symbols as in Fig. 2.

increases until 36 hours and then decreases, and with 5 per cent. it increases continuously throughout the experimental period.

The potassium effect is highly significant. On the other hand, the interaction between potassium and sugar is also significant. The data indicate

TABLE VIII

*Effect of nutrient treatment on respiration (ml. O₂/20 segments/hour).
Symbols as in Table III*

Hrs.	0		0.25		2.0		5.0	
	-K	+K	-K	+K	-K	+K	-K	+K
12	25.0	23.0	59.7	61.8	60.5	65.1	61.4	68.8
24	17.3	18.4	48.2	55.6	59.6	74.5	64.1	74.6
36	15.4	15.7	33.8	43.3	67.0	85.6	72.1	96.2
48	13.5	12.9	27.6	39.7	62.4	78.1	76.6	99.0

Statistical analysis

Variance due to:	D.F.	Mean square	V.R.	
Time	3	11.3	1.63	—
Potassium	1	685.43	98.77	HS
Sugar	3	5,627.67	810.90	HS
K × C	3	105.29	15.17	HS
T × C	9	216.03	24.00	HS
T × K	3	42.86	6.18	S
Error	9	6.94	—	—

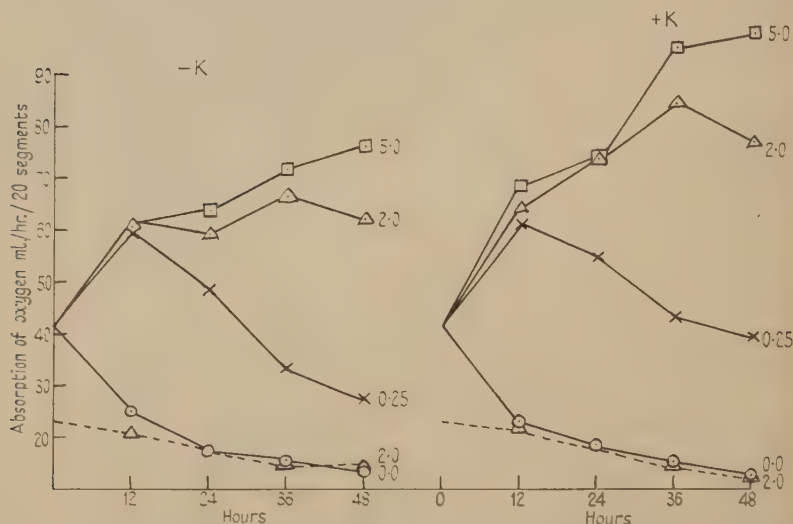


FIG. 7. Change in respiration rate with time with different treatments.

Symbols as in Fig. 2.

that potassium stimulates respiration only in the presence of added sugar. In the absence of sugar, potassium evidently has no effect. The significant interaction between potassium and time suggests that the stimulating effect of potassium is greatest in the later phases of the experimental period.

The data of Table VIII do not show one feature which is evident from the curves of Fig. 7. Whereas with water respiration decreases from the beginning of the experiment, with 0.25 per cent. sugar it increases during the first 12 hours and then decreases. Moreover, an inspection of the curves for 0.25 per cent. sucrose shows that although the respiration rate is decreasing during the second 12-hour interval, nevertheless the average rate during this interval is greater than it is during the first. During the third interval the average rate is clearly lower than it is in the second.

Results obtained with an atmosphere containing 5 per cent. oxygen. All the data obtained with 5 per cent. oxygen and with 2 per cent. sucrose solutions are assembled in Table IX. Each set of values is presented graphically in one or other of Figs. 2-7.

TABLE IX

Results with 5% oxygen and 2% sucrose on extension E (mm./segment); dry weight DW (mg./20 segments); sugar absorption, SA (mg./20 segments); sugar content, IS (mg./20 segments); cellulose content, C (mg./20 segments); respiration, R (ml. O₂/20 segments/hr.). —K indicates absence and +K presence of potassium in medium

Hrs.	E		DW		SA		IS		C		R	
	—K	+K	—K	+K	—K	+K	—K	+K	—K	+K	—K	+K
12	2.6	2.5	4.54	4.46	0.86	0.79	0.89	0.98	1.23	1.21	20.9	21.8
24	2.9	2.8	4.49	4.24	2.6	2.7	1.05	1.13	1.39	1.32	17.1	19.1
36	2.9	3.0	4.30	4.45	3.8	3.7	0.97	0.99	1.08	0.98	15.2	14.5
48	2.9	2.9	4.39	4.49	4.31	4.5	0.99	1.03	1.29	1.18	14.2	13.6

A comparison of the data of Table IX with those obtained with the same 2 per cent. sugar media with air shows that extension, absorption, and respiration are all strongly depressed when the partial pressure corresponds to 5 per cent. of oxygen. Respiration instead of increasing decreases progressively with time. Whereas with air, dry weight, sugar, and the cellulose content increase with time, with 5 per cent. oxygen they remain more or less constant. It is significant that an effect of potassium is not given in any connexion with the low partial pressure of oxygen.

DISCUSSION

The changes that occur in the medium with respect to sugar are clearly of some importance in relation to the interpretation of the course of absorption and the relevant data are given in Table X.

Evidence is presented below which indicates that sugar is absorbed as monosaccharide, and the data of Table X therefore indicate that the tissues hydrolyse the sucrose more rapidly than the products are absorbed and this fraction accumulates in the medium. This phenomenon has previously been considered in some detail by Burström (1941) and Dormer and Street (1949). In the present connexion two aspects of the position may be noticed. The hydrolysis is not affected either by potassium or the partial pressure of oxygen, and differences in absorption that may occur with these variables

TABLE X

Reducing sugar (R) and sucrose (S) (mg./2 ml.) medium in different experimental series. Symbols as in Table III

Hrs.	0.25 (Air)				2.0 (Air)			
	-K		+K		-K		+K	
	R	S	R	S	R	S	R	S
	—	—	—	—	—	—	—	—
0	—	5.0	—	5.0	—	40.0	—	40.0
12	2.96	0.30	3.12	0.18	7.7	28.5	7.4	32.6
24	1.66	0.16	1.75	0.11	14.4	19.0	14.8	17.8
36	0.82	0.14	0.80	0.12	22.0	7.0	22.0	6.1
48	0.52	0.11	0.46	0.10	20.2	4.6	19.9	4.3

	5.0 (Air)				2.0 (5% O ₂)			
	-K		+K		-K		+K	
	R	S	R	S	R	S	R	S
	—	—	—	—	—	—	—	—
0	—	100.0	—	100.0	—	40.0	—	40.0
12	7.6	84.8	6.8	87.2	6.9	32.2	7.1	32.0
24	18.5	69.6	19.2	68.6	14.7	22.6	14.4	23.0
36	27.2	54.2	26.8	54.6	22.8	13.4	22.1	14.3
48	39.7	36.2	38.9	35.9	25.2	10.5	24.9	10.7

are therefore not due to corresponding differences in the availability of mono-saccharides in the medium.

With all media the total sugar content decreases with time, but the relative decrease is of course greatest in the least concentrated medium. With 0.25 per cent. sucrose the total amount of available sugar has been reduced at 36 hours to less than one-fifth of the original value. The concentration of hexose increases at first and then decreases as absorption proceeds. With 0.25 per cent. sucrose the decrease sets in at 12 hours and with 2.0 per cent. sucrose at 36 hours.

The sugar content. The data of Fig. 6 show that when sugar is not provided in the medium the internal sugar content tends to decrease with time, and this is no doubt due to a consumption of the available reserves of sugar in respiration. When sugar is provided and the tissue is exposed to air absorption occurs, but the amount consumed in respiration is only a small proportion of the total amount absorbed, and of the residue a part accumulates in the vacuole. When the partial pressure of oxygen is depressed, all the sugar absorbed is apparently consumed in respiration and the internal content of sugar does not change.

An evaluation of the significance of the internal sugar content, however, requires that the relevant data should be expressed as concentrations, and calculated values for concentration are given in Table XI. These values are in terms of molar equivalents, and they represent the combined molar concentrations of reducing sugar (assumed to be hexose) and sucrose in the estimated water content of the tissue. As indicated above, in the determination

of the absolute sugar content reducing and sucrose fractions were measured separately. The water content has been estimated from the linear dimensions of the segments. Throughout the investigation, breadths as well as lengths have been measured, and from these measurements volumes have been calculated which have been taken as estimates of water content. In Table XI the molar equivalent concentrations for the medium corresponding to the internal values are also given together with, in certain instances, the differences and the ratios between the internal and external concentrations. The differences have been calculated since they are estimates of the osmotic gradient due to sugar and the ratios since they are estimates of accumulation.

TABLE XI

Concentration of sugar in molar equivalents in the external medium (E) and in the tissues (I)

0.25% sucrose (air)

Hrs.	-K				+K			
	E	I	I-E	I/E	E	I	I-E	I/E
0	0.007	0.087	—	—	0.007	0.087	—	—
12	0.008	0.067	0.059	8.0	0.009	0.051	0.42	5.7
24	0.005	0.084	0.079	16.8	0.005	0.052	0.047	10.4
36	0.002	0.095	0.093	47.5	0.002	0.068	0.066	34.0
48	0.002	0.091	0.089	45.5	0.001	0.062	0.06	62.0

2.0% sucrose (air)

Hrs.	-K				+K			
	E	I	I-E	I/E	E	I	I-E	I/E
0	0.056	0.087	—	—	0.056	0.087	—	—
12	0.061	0.093	0.032	1.5	0.066	0.080	0.014	1.2
24	0.066	0.125	0.059	1.9	0.066	0.177	0.051	1.8
36	0.071	0.159	0.088	2.2	0.069	0.135	0.066	2.0
48	0.063	0.176	0.113	2.8	0.061	0.144	0.083	2.4

5.0% sucrose (air)

Hrs.	-K				+K			
	E	I	I-E	I/E	E	I	I-E	I/E
0	0.139	0.087	—	—	0.139	0.087	—	—
12	0.139	0.240	0.101	1.7	0.140	0.200	0.060	1.4
24	0.148	0.195	0.147	1.3	0.148	0.183	0.035	1.2
36	0.152	0.222	0.070	1.5	0.150	0.207	0.057	1.4
48	0.160	0.296	0.136	1.8	0.158	0.257	0.099	1.6

2.0% sucrose (5% oxygen)

Hrs.	-K		+K	
	E	I	E	I
0	0.058	0.087	0.058	0.087
12	0.064	0.066	0.064	0.071
24	0.072	0.072	0.072	0.062
36	0.082	0.072	0.081	0.065
48	0.085	0.065	0.084	0.069

Water (air)

Hrs.	-K		+K	
	E	I	E	I
0	0.0	0.089	0.0	0.087
12	0.0	0.056	0.0	0.058
24	0.0	0.047	0.0	0.044
36	0.0	0.037	0.0	0.041
48	0.0	0.026	0.0	0.029

It is probable that most of the free sugar is in the vacuole, and the relevant data of Table XI may therefore be considered in terms of concentrations in the vacuolar sap. It is evident that when sugar is provided and the cultures are exposed to air the internal sugar concentration increases with time and with concentration of sugar in the external medium. Further, that after extension begins the internal concentration is always higher than the external, and the ratio between the two is always greater than one. The value of the ratio, it may be noted, increases with time and decreases with increase in concentration in the external medium. In relation to the effects of the several media on growth it is of some significance that the difference in concentration between the internal and external solutions is not strikingly affected by the concentration of the external medium. The increase tends to become greater as the concentration of the external medium increases, although the effect is clearly small and probably negligible.

In the presence of potassium the concentration in the sap is markedly depressed. This is a result of a decreased absolute content of sugar with an increased water content. It is significant, however, that the potassium effect on internal sugar concentration is only given when the cultures are provided with sugar and exposed to air.

Absorption. Although absorption continues at a constant rate with the two high sugar media, with the 0.25 per cent. solution the rate decreases continuously after about the first 12 hours. This is clearly a result of the rapidly decreasing concentration in this solution shown by the data of Table XI.

During growth the absolute amounts of both sucrose and reducing sugar in the tissue increase, the increase being relatively greatest with the reducing fraction. This suggests that sugar is absorbed as hexose, and is subsequently converted into sucrose in the cell. The increase in the absolute amount of reducing sugar in the cell leads, however, to a concentration of the reducing fraction which is several times greater than the concentration of the corresponding fraction in the external solution. This is shown by the data of Table XII, which are ratios between the internal and external concentrations of reducing sugar for the series provided with sucrose and exposed to air.

TABLE XII

*Ratios between internal and external concentrations (I/E) of reducing sugar.
Symbols as in Table III*

Hrs.	0.25		2.0		5.0	
	-K	+K	-K	+K	-K	+K
12	6.1	4.0	3.3	2.8	8.7	6.8
24	14.8	9.6	2.9	2.4	3.1	3.0
36	41.5	28.0	2.4	2.0	2.3	2.1
48	77.0	55.0	2.8	2.4	2.1	1.8

The high values given with the 0.25 per cent. medium towards the end of the experimental period are due to the rapid decrease in concentration in the medium, as well as to the increase in concentration in the vacuolar sap. What-

ever the nature of the medium, however, the value of the ratio is always greater than one. This suggests that the absorption of sugar occurs against a concentration gradient, and that a transport mechanism is therefore involved in absorption. Although the ratios do not provide decisive evidence for this conclusion since the compositions of the reducing fractions are not known, other evidence is available which is consistent with this interpretation. When the partial pressure of oxygen is reduced, absorption of sugar is depressed. This is clearly not an indirect effect of the partial pressure of oxygen on the absorption of water. If it were, then absorption should lead to a high concentration of sugar in the tissue. The data of Table XI show that in fact the concentration with 5 per cent. oxygen is considerably lower than it is in air. Clearly the low level of oxygen immediately influences sugar absorption. The data of Table IX indicate that with 5 per cent. oxygen respiration is decreased, and the evidence therefore suggests that the absorption of sugar depends on a transport mechanism which involves the consumption of respiratory energy.

Most of the sugar in the cell is probably in the vacuole and a large proportion of the sugar absorbed is undoubtedly released into it, but with respect to the movement into the vacuole it is probable that at least two distinct stages are involved, the movement into the protoplast and the subsequent movement from the protoplast into the vacuole. When the cultures are exposed to an atmosphere containing 5 per cent. oxygen the internal sugar concentration remains lower than the external (Table XI). It is improbable that the resistance to diffusion is increased when the supply of oxygen is restricted, and the position observed with the low partial pressure of oxygen suggests that the permeability of the protoplast or of some component of it to sugar is low. On the other hand, evidence discussed below indicates that when sugar is not provided in the medium, respiration depends on sugar which diffuses from the vacuole into the protoplast. Thus it is probable that the barrier to free diffusion is on the external surface of the protoplast. If such is the case, then the transport mechanism must operate at least across this surface. It may also operate across the whole protoplast, but it is evident that sugar does diffuse fairly readily between the protoplast and the vacuole and certain results discussed below are certainly compatible with the interpretation that sugar is transported into the protoplast and subsequently diffuses into the vacuole.

Respiration. When sugar is not supplied and the tissue is exposed to air the respiration rate decreases with time, and the data of Table VII suggest that this is due to a progressive decrease in the substrate level in the tissue. This decrease in sugar concentration is itself occasioned by the continuous consumption of sugar in respiration, and the evidence suggests that the consumption in the protoplast promotes a diffusion from the vacuole which becomes progressively less rapid as the concentration in it decreases.

Respiration is also depressed when the segments are exposed to an atmosphere containing 5 per cent. oxygen. This treatment also occasions a low absorption, and this has been attributed to the low respiration rate. The converse possibility may be considered, however, that the respiration may be

determined by the absorption. In this connexion the respiration rate established immediately after the tissue is transferred to the experimental atmosphere is of some significance. If absorption determines the position, then respiration might be expected to change as it does with air when sugar is not provided. In fact, whereas with air in the absence of sugar respiration is high initially and decreases with time, with a low partial pressure a low rate is established immediately (Fig. 7). Thus it is evident that the depressed respiration is not an indirect effect of a low absorption.

When air is supplied to the cultures and sugar is provided, the respiration rate increases with increasing concentration of sugar in the medium, and this may be attributed to the corresponding increase in the internal level of sugar in the tissue which is determined by the increasing absorption.

The changes in respiration rate with time in the several sugar media may be interpreted in terms of relative rates of accumulation in the protoplast and of dispersal into the vacuole. The immediate effect of placing the tissue in the sugar solution is a rapid rise in the respiration rate. With the 0.25 per cent. medium during the first 12 hours this rise occurs while the concentration in the tissue is decreasing. It may be suggested that the rate of delivery into the protoplast is relatively greater than the rate of diffusion into the vacuole, and as a result the immediate effect of placing the tissue in a sugar solution is to promote an accumulation in the protoplast with a consequent increase in respiration. If water is absorbed simultaneously with the sugar and the absorption of water is relatively more rapid than that of sugar, then with a local accumulation in the protoplast the overall concentration may decrease, while the concentration in the protoplast and consequently the respiration are increasing. With the 0.25 per cent. solution again, although absorption of sugar continues after 12 hours and the overall concentration continues to increase, nevertheless the respiration rate decreases progressively with time. On the other hand, although absorption continues, the rate of it decreases and it is therefore probable that after the initial rise in concentration in the protoplast this decreases as a result of a decreasing rate of absorption which does not compensate for the diffusion of sugar into the vacuole.

With the 2.0 and 5.0 per cent. solutions the rate of respiration continues to increase after 12 hours. With these two solutions the rate of absorption remains high and constant, and respiration increases, no doubt as a result of a continually increasing concentration in the protoplast, but in this connexion the increased protoplast concentration may not be accompanied by a relatively lower concentration in the vacuole. The present series of results and certain others which are not given here suggest that while absorption continues throughout the experimental period, respiration with the 2.0 per cent. medium decreases after 36 hours. The significance of this, however, cannot be elucidated from the present series of data.

Potassium has a marked stimulating effect on respiration, but only in the presence of sugar and when air is provided. The stimulation is clearly not due to an effect on the mechanism of oxidation, since if it were, then some stimula-

tion might be expected when sugar is not provided. It is evidently not due entirely to an effect on absorption. There may be a slight enhancement of absorption with potassium, but if the whole stimulation to respiration were due to an enhanced absorption, then an increased concentration of sugar in the tissue might be expected and this is never observed. In terms of the mechanism of sugar movement proposed above, it may be suggested that potassium decreases the permeability of the protoplast to sugar. As a result, the rate of diffusion into the vacuole is decreased, and with more or less the same rate of absorption the concentration in the protoplast and with it the respiration is increased. This interpretation of the potassium effect in respiration is consistent with the observation that potassium depresses the absolute content of sugar. Clearly the enhanced respiration without a corresponding effect on absorption involves a larger proportion of the total amount absorbed being consumed in respiration, and a smaller increment being contributed to the total sugar content. The interpretation is also consistent with the observation that the potassium effect is not given when the partial pressure of oxygen is low. If the effect develops through a change in the permeability of the protoplast to sugar, then it can only occur when there is a comparatively rapid transport of sugar into the vacuole. This condition is not established with subnormal concentrations of oxygen since with these the rate of absorption is depressed, and with these again the amount of sugar in the protoplast is not such that its concentration can be appreciably altered by a change in permeability.

Dry weight. The absorption of sugar leads to increases in respiration, internal sugar content, and cellulose, and the second and third contribute to corresponding increases in dry weight. It is evident from the dry weight data, however, that sugar may be involved in other contexts than those considered here. This is indicated by the differences between dry weight increases and the combined increases for sugar and cellulose which are always the smaller.

Cellulose content. In the absence of sugar in the medium and when the segments are exposed to air, the cellulose content decreases with time, which suggests that with starvation conditions cellulose components of the wall may be hydrolysed. When the oxygen content of the atmosphere is reduced to 5 per cent. the cellulose content does not change, and this is probably an effect of the low absorption which is occasioned by this treatment. When sugar is provided a rapid synthesis of cellulose occurs, the rate of which remains more or less constant with time. The rate, moreover, increases when the concentration in the medium is raised from 0.25 to 2.0 per cent. Further increase in concentration, however, has little effect, which suggests that above 2.0 per cent. the rate may be limited by that of an enzyme reaction.

Although cellulose increases, it does not increase at the same rate as length. This is emphasized by the values of Table XIII, which are ratios between the cellulose content and length, and are measures of the quantity of cellulose per unit area of cell surface. Below, these ratios are referred to as measures of thickness, but clearly they are such only in the sense defined.

TABLE XIII

*Ratios between cellulose content and length of segment.**Symbols as in Table III*

Hrs.	0.25 (air)		2.0 (air)		5.0 (air)		2.0 (5% O ₂)		Water (air)	
	—K	+K	—K	+K	—K	+K	—K	+K	—K	+K
0	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
12	0.34	0.31	0.37	0.39	0.50	0.40	0.49	0.49	0.34	0.36
24	0.31	0.25	0.44	0.43	0.45	0.40	0.50	0.47	0.27	0.24
36	0.37	0.28	0.57	0.57	0.58	0.59	0.39	0.36	0.22	0.24
48	0.39	0.31	0.72	0.73	0.76	0.74	0.44	0.41	0.19	0.21

In most series thickness decreases sharply during the first 24 hours. With water and the cultures exposed to air this decrease continues, since in addition to the stretching effect of extension during the first 12 hours, in this series components are removed from the wall by hydrolysis throughout the experimental period. With the low partial pressure of oxygen the initial decrease is not followed by a recovery since with this treatment there is no synthesis of cellulose. When sugar is supplied and the cultures are provided with air, however, the decrease is followed by a progressive increase in thickness during the final 24 hours, and this suggests that at least during the final phase growth is not being limited by the rate of cellulose formation.

It may be noted that since the values for 48 hours are not greater than those for the untreated segment, it is probable that a secondary wall is not formed and that during the whole experimental period the wall grows by intussusception.

Growth. When sugar is not provided in the medium, growth is limited. The absorption of water with this treatment is accompanied by a rapid increase in wall pressure as the wall is stretched and by a rapid decrease in the osmotic pressure of the vacuolar sap. A stage is reached in a comparatively short time when the wall pressure balances the turgor pressure and at that stage growth ceases. Since the absorption of water is probably relatively more rapid than that of sugar, it is also probable that immediately after the tissue is placed in the experimental fluid the rate of water absorption and therefore the rate of extension is highest when water only is supplied. In sugar solutions, however, the time of extension is prolonged and the rate with sugar exceeds that with water within a comparatively short time.

Growth is increased when sugar is provided, and the rate of extension tends to increase with increasing concentration in the external medium. The concentration of sugar in the medium, however, also affects the rate of cellulose formation, the rate of sugar accumulation in the vacuole, and the rate of respiration. Clearly the effects on growth are due to primary effects on these processes. In the conditions of the present series of experiments the extension of the wall only occurs to a limited extent with simple stretching and the continual increase in the area of the wall evidently depends on the formation of structural components which are incorporated into it. Similarly,

the amount of water that can be absorbed with the quantity of solutes originally present in the segments is limited. Thus it is clear that the fourfold increase in length that occurs in some of the sugar media depends at least partly on cellulose formation which provides for the continual expansion of the wall and on the accumulation of sugar in the vacuole which promotes the absorption of water by maintaining a high osmotic pressure in the vacuolar sap. The increasing growth rate with increasing sugar concentration in the medium may therefore be attributed (again at least partly) to an increasing absorption which provides progressively greater quantities of sugar for cellulose formation and simultaneously for sustaining water absorption through the accumulation of solutes in the vacuole.

It may be noted in this connexion that while the rate of extension with 5.0 per cent. sucrose is higher than it is with 2.0 per cent. after 12 hours, it clearly is not so before this time. When the tissue is placed in the 5.0 per cent. solution the sugar concentration in the sap is lower than it is in the medium and it is probable that water absorption is retarded until the concentration of sugar has been raised in the sap.

Since the rate of extension evidently depends to some extent on the rates of cellulose formation and on the rate of sugar accumulation in the vacuole, and since these depend on the rate of absorption which in turn depends on respiration, then clearly respiration must influence growth to the extent that it determines sugar absorption. Undoubtedly the depressed growth with 5 per cent. oxygen is due to a low respiration which limits sugar absorption, which in turn restricts wall formation and the accumulation of solutes in the vacuole. There may, however, be other connexions in which respiration influences the growth rate. With the 0.25 per cent. medium, growth ceases after 24 hours, although after this time cellulose formation continues and as indicated above the concentration of sugar in the vacuole continues to increase. On the other hand, the average respiration rate for each 12-hour interval increases until 24 hours and then decreases. Thus the evidence suggests that growth ceases as a result of the decreasing respiration rate. Further, with the 2.0 per cent. medium growth ceases and respiration begins to decrease at 36 hours, while with the 5.0 per cent. solution in which growth continues throughout the experimental period respiration also increases continuously.

The data of Fig. 2 show that potassium has a strong stimulating effect on growth, but they also show that it only has this effect in circumstances in which it also stimulates respiration. Potassium has no effect on either growth or respiration when sugar is not provided and when the cultures are exposed to an atmosphere containing 5.0 per cent. oxygen, but it does stimulate both when the medium contains sugar and when air is available to the tissue. The evidence suggests that the effect on growth is due to a primary effect on respiration. It has been shown above that with potassium the concentration of sugar in the tissue is considerably lower than it is without it. With this condition an enhanced growth might occur if the resistance of the wall to extension is reduced with an enhanced rate of cellulose formation, or if the reduction in the

concentration of sugar is compensated by a corresponding increase in the concentration of some other solute, or if respiration stimulates a non-osmotic process of water absorption. The first possibility may be disregarded since in the 0.25 per cent. sugar medium there is no effect of potassium on cellulose formation, although with this medium the effect on growth is large. An increase in the concentration of some solute other than sugar is also not probable. It is possible that the enhanced respiration which is due to potassium promotes the accumulation of potassium and chloride ions in the vacuole, but if the effect of potassium on growth is due to such a mechanism, then some effect of potassium on growth should be given in the absence of sugar. The third possibility that potassium stimulates a non-osmotic absorption mechanism through an effect on the respiration rate is being investigated experimentally.

It may be emphasized that although the present series of data show how various treatments may affect the rate of extension, they do not show how these treatments affect the time of extension.

SUMMARY

1. A new technique for studying extension growth in the root is described which is based on excising a zone which extends 1.5–3.0 mm. from the tip. Large numbers of these segments are cultured with different nutrient fluids in the dark at 25° C. with continual shaking.

2. The effects of a large number of nutrients on the growth of the segments have been studied, but only two, sugar and potassium ions, have been found to have stimulating effects.

3. The effects of water, three concentrations of sugar, and one of potassium in air, and with an atmosphere containing 5 per cent. oxygen have been studied in detail in connexion with length increase, sugar absorption, content of free sugar, cellulose content, dry weight, and respiration.

4. It has been shown that with increasing concentration of sugar in the medium, the rate of growth, the time during which growth proceeds, the internal concentration, respiration, dry weight, and cellulose formation all increase. Also that potassium stimulates the rate of growth and respiration, and that with 5 per cent. oxygen all the aspects studied are depressed.

5. It is suggested that the stimulation due to sugar may be attributed to an acceleration of water absorption with a complementary increase in cellulose formation. It is further suggested that sugar accelerates water absorption by accumulating in the vacuole and thus sustaining the osmotic pressure of the vacuolar sap. It is further suggested that potassium stimulates growth by increasing water absorption through an effect on respiration. The effect of respiration in this connexion may be to promote the transport of water directly or to enhance the osmotic pressure of the sap by inducing an accumulation of inorganic ions in the vacuole.

LITERATURE CITED

- BONNER, J. (1933). *J. Gen. Physiol.* **17**, 63.
 — (1934). *Proc. Nat. Acad. Sc.* **20**, 393.
 BROWN, R., and RICKLESS, P. (1949). *Proc. Roy. Soc., B*, **136**, 110.
 BURSTRÖM, H. (1941). *Ann. Agric. Coll. Sweden*, **9**, 264.
 — (1942). *Ibid.* **10**, 209.
 — (1947). *K. fysiogr. Sällsk. Lund Forh.* **17**, 1.
 CZAPEK, R. (1922). *Biochemie der Pflanze*. Jena.
 DORMER, K. J., and STREET, H. E. (1949). *Ann. Bot.*, **13**, 199.
 SCHNEIDER, C. L. (1938). *Amer. J. Bot.* **25**, 258.
 STREPKOV, S. M. (1937). *Zeit. f. anal. Chemie*, **111**, 57.
 THIMANN, K., and SCHNEIDER, C. L. (1938). *Amer. J. Bot.* **25**, 259.
 — (1948). *Ibid.* **35**, 271.

Studies in Stomatal Behaviour

IV. THE WATER-RELATIONS OF THE EPIDERMIS¹

W. T. WILLIAMS

Bedford College for Women, London

INTRODUCTION

THE remarkable observations of La Rue (1930) on the water-relations of the epidermis of Angiosperm leaves appear to be less widely known than they deserve. He established that either or both of the epidermes may be detached from the underlying mesophyll in one of three ways: (i) naturally, as in *Mitchella repens*, in which the lower epidermis (with the hypodermis) in all except the youngest leaves becomes detached from the mesophyll except at the leaf margins and over the midrib (a similar condition, but affecting only small areas of epidermis, was observed in a number of other Angiosperm species); (ii) by the activities of leaf-mining insects; and (iii) artificially, by the insertion of a fine glass probe, a technique particularly successful with *Saxifraga sarmentosa*. In all cases he found that the epidermis remained healthy for at least 3 weeks, though as might be expected the area of detached epidermis which could be so maintained differed somewhat in different species. He therefore reasonably concluded that the epidermis does not rely for its water-supplies on the underlying mesophyll; and in the case of *Saxifraga sarmentosa* he was able, by vaselining the inner surface of the detached epidermis, to show that it is not even dependent on water-vapour obtained from the presumably saturated intercellular atmosphere of the leaf. The water-supply appears in fact to be obtained primarily from the main veins, and to be disseminated solely by lateral diffusion through the cells of the epidermis.

La Rue made no stomatal observations; but Thoday (1938) reported that in the case of *Kleinia articulata* and *Sedum album* the stomatal behaviour appeared to depend more on the humidity of the external atmosphere than on the water-content of the leaf. This, as he pointed out, is entirely compatible with La Rue's findings; but it suggests further that not only does the epidermis not normally require water-vapour from the intercellular atmosphere, but that it is unable to take up such water-vapour when it is in fact needed. The matter was raised again by Spencer (1938), who attempted to evaluate the relative importance of the effects of humidity and movement of air upon the stomata. However, the importance of the CO₂-content of the air within a porometer cup (Heath and Williams, 1948; Heath, 1948; Williams, 1949) was not at that time realized, and the effect of using dry air was masked by the closing effect due to sweeping the cup with air of normal CO₂-content. Spencer

¹ Earlier papers of this series: Williams, W. T. (1948), *Ann. Bot.*, N.S. **12**, 35-51; Heath, O. V. S. (1949), *New Phytol.* **48**, 186-211; Williams, W. T. (1949), *Ann. Bot.*, N.S. **13**, 309-37.

therefore rightly concluded that, with a porometer cup as normally set up, humidity was of negligible importance compared with movement.

In the course of the investigations by Heath and the present author into the cause of the wide opening of stomata under a porometer cup, the effect of introducing a drying agent such as anhydrous calcium chloride or silica gel into the cup was noted. Although, as already reported, the wide opening was not thereby prevented, certain abnormalities in behaviour of the stomata under such cups were observed, which were considered to merit this further brief investigation.

MATERIAL AND METHODS

The plant used in all cases was *Pelargonium zonale*, var. 'Paul Crampel'; this is particularly suitable since, as a result of the researches of Pearse (1935), Spencer (1938), and Mer (1940), a considerable amount of information is available on the interrelationships of leaf water-content, transpiration, and stomatal behaviour in this variety. The porometer was a conventional form of simple resistance porometer (Gregory and Pearse, 1934) with a negative-pressure aspirator; the porometer cups were similar to those used by Spencer (but with the side-tubes blocked), so that a drying agent could be introduced into the cup. The manometers were read by eye at suitable intervals, and manometer pressure (i.e. the quantity P_2 of Gregory and Pearse) used as a measure of stomatal resistance, decreasing pressure representing stomatal opening. In the graphs which illustrate this paper, ordinates represent negative manometer pressures in cm. of dekalin (s.g. = 0.89), with a possible maximum pressure (P_1) of 12 cm.

EXPERIMENTAL RESULTS

1. Accelerated stomatal closure

This is the most characteristic phenomenon shown by stomata in a dry atmosphere, and is well exemplified by the following results:

Experiment 1. Two porometer cups were attached to an illuminated leaf still attached to the plant, one cup being filled with water, the other with anhydrous calcium chloride; the resulting records are shown in Fig. 1 (a). The behaviour of the two sets of stomata is at first identical, each showing the typical Knight-effect 'recovery' curve. At the point x on the record the cups were darkened by placing a halfpenny over each; and it will be seen that, even after this relatively short exposure to the drying agent, the 'dry' stomata show a markedly increased rate of closing. Fig. 1 (b) shows the even more striking result of a similar experiment, in which the two cups remained attached and darkened overnight. At the beginning of the record both cups were illuminated, and both open normally; at the point x both were darkened as before, with the remarkable result shown. It must be stated that it has not been possible to correlate the extent of the acceleration with the time of exposure to the drying agent; differences in acceleration seem rather to reflect

differences in behaviour of individual leaves or perhaps plants, and are not unlike the wide variations in closing rate met with in normal porometer cups. At the point *y* in Fig. 1 (*b*) the cups were again illuminated, and normal opening follows.

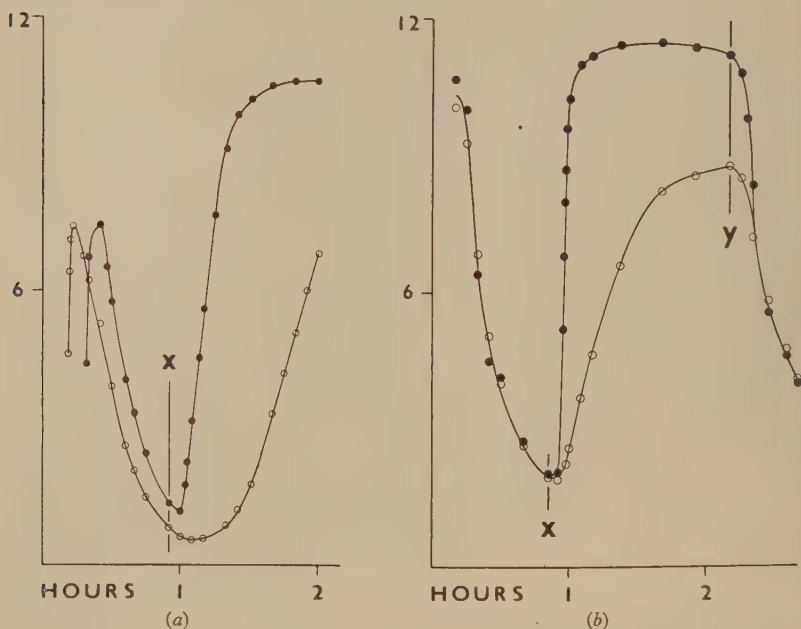


FIG. 1. Expt. 1. Accelerated stomatal closure. Open circles, 'wet' cup; closed circles, 'dry' cup.

Experiment 2 was carried out on a detached leaf with its petiole dipping into tap-water. Two cups, one containing water and the other calcium chloride, were attached and darkened overnight as before. The records are shown in Fig. 2, in which Record *A* represents the group of stomata at first under the 'dry' cup, and continues to represent this same group of stomata throughout. At the beginning of the experiment the leaf was illuminated and the stomata opened normally; at the point *x* the cups were darkened, and the expected accelerated closure is shown by the stomata under the 'dry' cup. The leaf was again illuminated and the stomata allowed to open for 100 minutes; this period of illumination is represented by the first break in the record, the pressure attained at the end of the period being shown at the beginning of the second part of the record.

At this point the cups were interchanged. The stomata of Group *A*, which had been 'dry' and were now 'wet', show a marked temporary closure; those of Group *B*, which had been 'wet' and were now 'dry', show practically no such closure. The mechanical shock was, so far as is known, of the same order in the two cases. Now Heath (unpublished; personal communication) has

shown that the closing response brought about by an increase in CO_2 -concentration is similarly accelerated under a 'dry' cup. The interchange of the cups will temporarily bring about such an increase, since for a few minutes the stomata will be exposed to ordinary laboratory air; and the possibility of

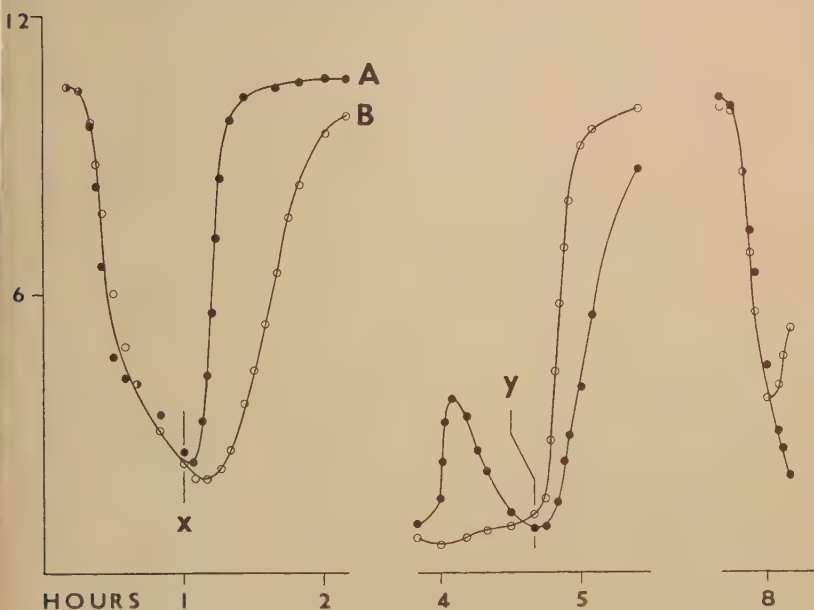


FIG. 2. Expt. 2. Accelerated stomatal closure; for explanation see text.

a small shock-induced respiratory increase (Audus, 1939) cannot be ruled out. This behaviour, then, is in accordance with expectation.

After 50 minutes (point *y*) the cups were again darkened, and it will be seen that the properties of the two groups of stomata have interchanged in accordance with the interchanged external conditions. This is important, since it precludes any possibility that the results might have been due to an inherent difference between the two sets of stomata. (The third part of the record, about 8 hours from the start, refers to a different type of experiment and will be considered in Section 4 below.)

No experiment of the general type of Expt. 1 has failed to elicit the phenomenon of accelerated closure, which may therefore be regarded as a normal response of *Pelargonium*. There remained the remote possibility that the effect was due to some specific property of calcium chloride, and check experiments were therefore carried out using phosphorus pentoxide and 'anhydrone' (anhydrous magnesium perchlorate); similar results were obtained, and the effect may therefore reasonably be attributed to the exposure to a dry atmosphere.

2. *Extent of closure*

Experiment 3. It is clearly desirable to ascertain whether the stomata under the 'dry' conditions ultimately close farther in the dark than do those under 'wet' conditions. Table I shows the results of five replicate experiments; in each case two cups, one 'wet' and one 'dry', were attached and darkened overnight, the values representing negative manometer pressures attained by the following morning. In all cases except (i) the leaf was attached to the plant.

TABLE I

			'Wet'	'Dry'
(i)	.	.	10.46	10.46
(ii)	.	.	11.36	11.62
(iii)	.	.	9.60	10.44
(iv)	.	.	8.20	10.30
(v)	.	.	8.54	10.80

There is undoubtedly a tendency to increased closure under the 'dry' cup, and in no case has the writer observed the greater closure to occur under the 'wet' cup; but it must be realized that these results refer to periods of darkening of from 12 to 18 hours, and that, owing to the very different *rates* of closure, observations taken after only a few hours of darkening might well show strikingly different *extents* of closure. It was also noticeable that as the 'wet' stomata approached the closed position their movements became extraordinarily sluggish, and a satisfactory equilibrium position never seemed to be attained. As Thoday observed for his succulent plants (unpublished; personal communication), stomata in a saturated atmosphere appear to be incapable of complete closure in darkness.

3. *The effects on stomatal opening*

Normal stomatal opening, after the first few minutes, is very rapid over a large part of its course; and unless abnormal conditions produce a marked *decrease* in rate of opening, or unless readings are taken at very short intervals, variations in the opening movement are somewhat difficult to evaluate. However, although the opening movements of both 'dry' and 'wet' stomata follow substantially the same curve, a closer examination of the records shows that in nearly every case the opening movement of the 'dry' stomata differs from that of the 'wet' in two respects: (i) it is slightly faster, and (ii) it tends to be irregular. Careful inspection of the first parts of the records of Expt. 1 (Fig. 1 (b)) and Expt. 2 (Fig. 2) will show that both these effects are present. It may be noted in this connexion that this irregularity may in rare cases be much accentuated, resulting in an irregular slow fluctuating movement of considerable amplitude; this has been observed on two occasions only (in each case a 'wet' cup on the same leaf was showing normal behaviour), and, since the phenomenon is shown by leaves in which part of the vascular system has been artificially interrupted (see Section 5 below), it possibly

means no more than that the water-relations of the leaf in question have become impaired in some way that has escaped notice.

The extent of opening has been compared both by the porometer and by Lloyd's stripping method. Examination of strips gave the impression that the stomata opened to a very slightly greater extent under the 'dry' cup; but the porometer results did not confirm this observation, and it is clear that the difference, if any, must be very small. At first sight this might appear to conflict with Thoday's (1938) statement that '... a turgid leaf in a dry atmosphere will open its stomata less widely than a leaf with a considerable water deficit in a saturated atmosphere'. However, Professor Thoday has kindly made available to the writer a more detailed account of his experiments, from which it is clear that the results obtained in a saturated atmosphere in light are likely to have been complicated by the wide-opening effect due to CO₂ deficiency; the discrepancy can therefore be disregarded at present.

4. *The effect of wilting*

At the end of the second part of the record of Expt. 2 (Fig. 2) the leaf was left in darkness for an hour; the water into which the petiole dipped was then replaced by a molar solution of sucrose in tap-water, and the leaf left in darkness for a further 75 minutes. The leaf was then illuminated, the result being shown in the third part of the record. Both sets of stomata start to open normally; but after about 20 minutes those under the 'dry' cup begin, somewhat abruptly, to reclose in the light. It was decided to investigate this phenomenon further.

Experiment 4. Two cups, one with water, the other with calcium chloride, were attached overnight. The following morning the petiole was cut under water, allowed to recover for half an hour, then, at the beginning of the record in Fig. 3 (a), darkened. At the point *x* on the record the water was replaced by molar sucrose solution and the leaf illuminated. It will be seen that as before both sets of stomata at first open normally; but that after about 25 minutes the stomata under the 'dry' cup begin, again rather abruptly, to close. The leaf was then darkened overnight and illuminated the following morning, with the result shown in the second part of the record. The 'wet' stomata still retain an appreciable power of movement, but the 'dry' stomata are virtually immobilized in the closed position.

Experiment 5. Two cups were attached and darkened overnight as before, except that anhydrous was used in place of calcium chloride; the following morning the petiole was cut under water. On illumination both opened normally, the 'dry' ones rather more rapidly (Fig. 3 (b)); at the point *x* on the record the petiole was dipped into 10 per cent. agar just above its setting temperature. This is the method of wilting used by Spencer (1938). Both sets of stomata show the typical preliminary opening on wilting, and both later begin to close; but the 'dry' stomata are obviously far more seriously affected. At a later stage in the experiment the 'dry' cup was replaced by one filled with water and the petiole cut again under water; but the stomata would not again

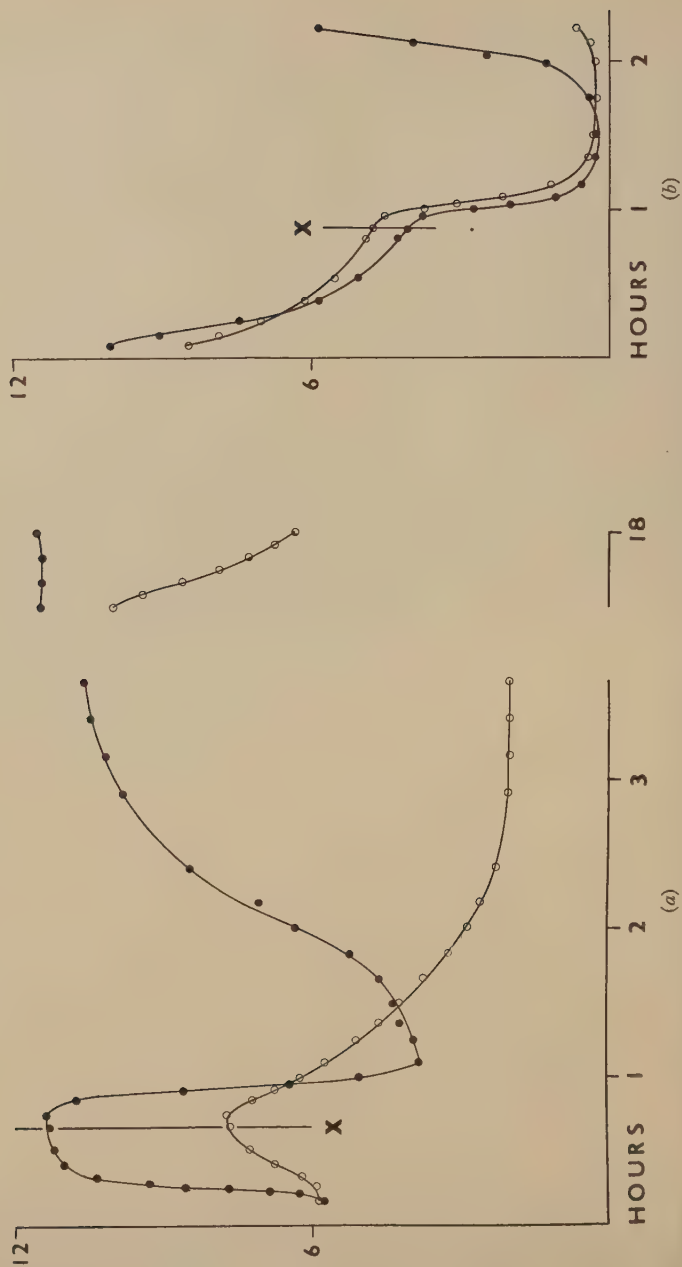


FIG. 3. The effect of wilting (a) in molar sucrose, (b) in agar. Open circles, 'wet' cup; closed circles, 'dry' cup. See text, Expts. 4 and 5.

open normally. It seems possible that stomata which have once closed in light as a result of a water-deficit suffer permanent damage; but no effect of comparable magnitude was reported by Spencer for his admittedly shorter periods of wilting, and the existence of such a 'damage' effect cannot be said to have been established.

5. *The effect of severing main veins*

Mer (1940) has shown that the bulk of the resistance to water-movement in the leaf of *Pelargonium* is that presented by the protoplasts of the mesophyll cells; in comparison with this the resistance to movement through the net-veins of the leaf is very small, and several main veins can be severed without discernible change in the suction-pressure or transpiration of the leaf. If, then, the epidermis can draw water from the mesophyll, severing the main veins serving a porometer cup (i.e. the two veins bounding the sector to which the porometer cup is attached) should have no effect; but if La Rue is correct in suggesting that the epidermal water-supply is drawn almost entirely from the main veins, severing those veins may well produce a local water-deficit in the epidermis. Since the loss of water from the epidermal cells (i.e. the cuticular transpiration) will be negligible if the surrounding air is saturated, it might be expected that the stomata in a 'wet' cup would be unaffected, whereas those in a 'dry' cup would show some effect similar to the familiar abnormal closure of wilting. One may note that an ordinary porometer cup, even without water inside, is in fact 'wet'.

Experiment 6. Two cups, one with water (*A*) and one with calcium chloride (*B*), were attached as usual. The veins shown in Fig. 4 were severed by a sharp pair of scissors, and a slip of vaselined cardboard inserted into each cut to ensure that there could be no appreciable passage of water across the cut. The leaf remained in the dark for 3 hours and was then illuminated, the resulting record being shown in Fig. 5. *A*, the 'wet' cup, shows normal opening; *B*, the 'dry' cup, shows an initial opening followed by an abnormal fluctuating movement of considerable amplitude. At the point *x* on the record the cups were interchanged, so that records *A* and *B* represent the same groups of stomata as before but with interchanged conditions of external humidity. *B*, which had been 'dry' and was now 'wet', shows as expected a vigorous temporary closure, but now followed by a normal opening; *A*, which had been 'wet' and was now 'dry', shows a smaller closure followed by a fluctuation similar to that originally shown by *B*. The leaf was darkened overnight and illuminated the following morning, and the second part of the record shows that these characteristics are still retained; the third part of the record shows substantially the same behaviour a few hours later.

The difference between the effects of wilting and severing the veins is important. A wilted leaf has lost its ultimate source of water, and, no matter by what route water is transported to the stomata, a time will come when the available water-supply is inadequate to maintain full turgor in the guard-cells, and the stomata close. Now we know from Mer's results (1940) that, even

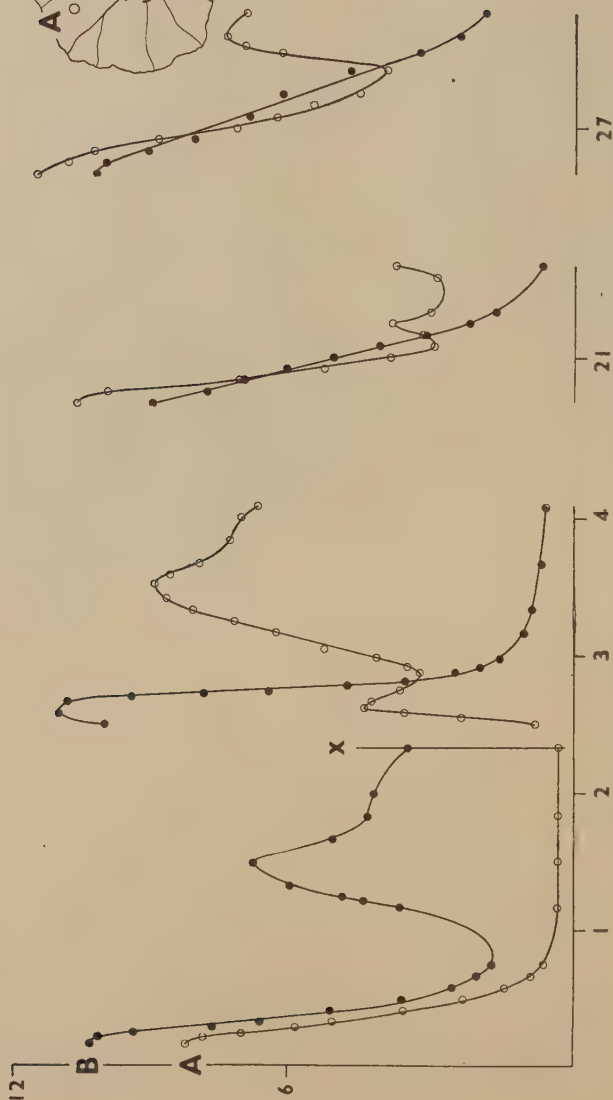


FIG. 5. Effect of severing main veins as shown in Fig. 4 (inset). For explanation see text, Expt. 6.

though main veins are severed, the mesophyll remains fully supplied with water; and the fact that the 'dry' stomata are showing a tendency to close suggests that the epidermis of the isolated part of the leaf is not supplied from the underlying mesophyll. The stomata do not show the uncompromising

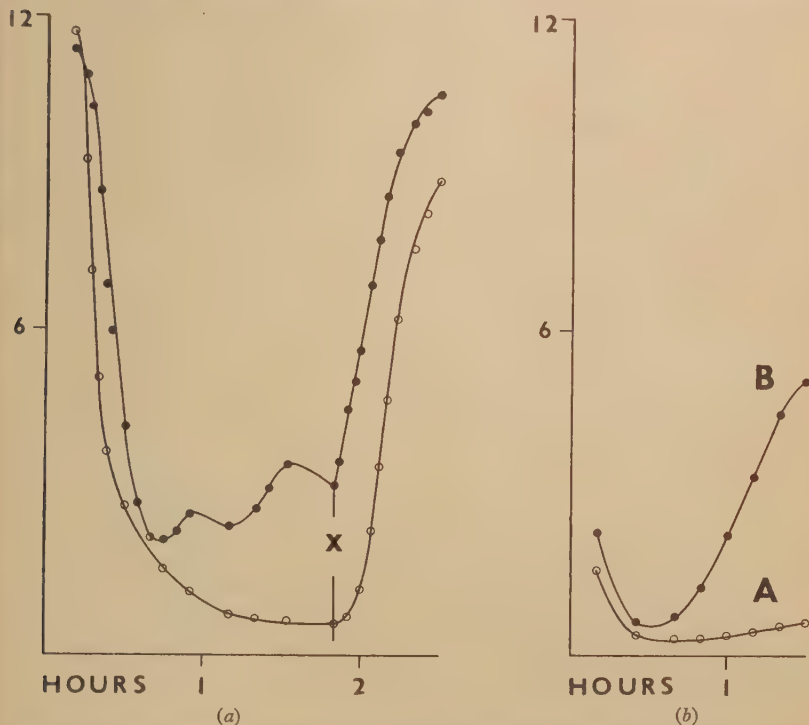


FIG. 6. Stomatal movements with both cups 'dry'. Closed circles, veins severed; open circles, control. (a) Expt. 7, (b) Expt. 8.

closure of wilting, but only an inability consistently to maintain full turgor in the light; this suggests rather that the effect of severance has been to interpose a resistance in the epidermal water-supply. On La Rue's hypothesis the epidermis is supplied from the parenchymatous tissue immediately below the main veins; and this may perhaps be incapable of attaining a suction pressure sufficiently high to draw an adequate supply of water, as would be necessary, from the mesophyll cells and the smaller veins of the lamina.

Experiment 7. In Expt. 6 the conditions within the two cups were different, but the veins serving both were severed; it was considered desirable to carry out an experiment in which the conditions within the two cups were the same (both 'dry'), but in which the veins serving only one cup had been severed. The result is shown in Fig. 6(a), in which the leaf was darkened at the point *x*, and it will be seen that the severance of the veins has had the expected effect.

Experiment 8 was a brief experiment combining the various forms of interference with the water-supply. Both cups were filled with calcium chloride, the veins serving cup *B* were cut, and at the beginning of the record the petiole, previously in water, was dipped into 10 per cent. agar, the leaf remaining illuminated. The result (Fig. 6 (*b*)) is as expected and calls for no comment.

DISCUSSION

1. *The data*

The behaviour of stomata under a porometer cup containing a drying agent, as compared with that of stomata under a cup containing water, may be summarized as follows:

- (i) They show markedly accelerated closure on darkening or in response to increased CO_2 -concentration.
- (ii) They show slightly accelerated and somewhat irregular opening on illumination.
- (iii) They are more rapidly and profoundly affected by interference with the water-supply to the lamina.

The discussion may conveniently be considered under the three headings which follow.

2. *The water-relations of the epidermis*

Whatever may be the precise nature of the mechanism responsible for the stomatal phenomena under discussion, it is clear that this mechanism is brought into action by exposure to a dry external atmosphere, with the consequent establishment of a water-deficit in either the guard-cells, the normal epidermal cells, or both. Even considering only their greater number and surface area, it seems unlikely that the normal epidermal cells would remain unaffected while a water-deficit was set up in the guard-cells; and in addition an appeal may be made to the anatomy of the average stoma, of which that of *Pelargonium* is a fair example. The outer and inner faces are heavily thickened, and least likely to be affected by humidity changes; the dorsal wall, being contiguous with the lateral wall of the neighbouring cell or cells, is not directly exposed to either the outer or the intercellular atmosphere; the only other thin area is the ventral wall which borders the stomatal pore, through which, as a 'pull' aspirator has been used, there passes a stream of air which may reasonably be supposed to be approaching saturation. We shall therefore postulate that exposure to a dry external atmosphere sets up a water-deficit in all the cells of the epidermis.

La Rue's observations leave no doubt that the epidermis is not normally dependent for its water-supply on the underlying cells or intercellular spaces of the mesophyll; the remarkable feature of the present observations is the implication that a serious water-deficit may be set up which *cannot* be relieved by the withdrawal of water or water-vapour from the mesophyll. It might be argued that the limiting factor is the *rate* of water-movement, i.e. that the

water loss into a permanently dry atmosphere is so rapid that water cannot be transmitted through the mesophyll cells at the necessary speed; but this is untenable. First, Mer has shown that even considerable interference with the vascular supply does not suppress transpiration, and Pearse and Spencer have shown that a wilted leaf, once again given access to a water-supply, recovers almost its full water-content in an extraordinarily short time. Secondly, neither Pearse, Spencer, nor Mer have reported any drop in water-content of the leaf as a result of stomatal transpiration into a dry atmosphere; in fact, provided there is an adequate water-supply to the leaf, such transpiration can apparently be continued indefinitely without damage to the mesophyll. Now the rate of loss of water from the mesophyll cells as a result of stomatal transpiration must be enormous compared with that lost through the cuticle; and if the mesophyll cells can mobilize water sufficiently rapidly to meet the one demand, they can undoubtedly meet the other.

The only reasonable conclusion appears to be that the inner surface of the epidermis presents, as does the outer, a formidable barrier to the passage of water or water-vapour. This has not usually been considered to be the case; indeed, Haberlandt (1914) states (p. 114): '... the inner walls of epidermal cells are generally thinner and softer than the outer walls, so that diosmotic interchange with the underlying tissues can proceed unhindered'. The significant 'generally' is not further enlarged upon except to consider cases of epidermes with mucilaginous inner walls. However, reference to an older and very reliable authority—de Bary (1884)—is illuminating; he states (p. 75): 'It (the cuticle) is wanting as a rule on the inner surface of the epidermis. It is rarely continued from the slits onwards over the whole inner surface of the epidermis, as far as this borders on intercellular spaces, as a lamella, which is interrupted by the surfaces of insertion of the subepidermal cells.' He then cites examples, and later (p. 77) mentions cases in which 'the whole wall of the epidermal cells is cuticularized all round'. Thin layers of cuticular material are notoriously difficult to demonstrate, and the possibility that an internal epidermal cuticle is a widespread phenomenon might perhaps justify the attention of the anatomists. Indeed, evidence of a related nature is already accumulating. Lewis (1948) adduces evidence in support of the contention that the mesophyll cells of many species have a hydrophobic surface; and Scott *et al.* (1947) state, again of a number of species, that 'In mature leaves a continuous suberin network outlines intercellular spaces and middle lamellae partially or completely. In half grown leaves the suberin films are at the limit of visibility.' Such a structure is entirely compatible with the results under discussion.

3. The water-relations of the guard-cells

Heath (1938) has confirmed the truth of the long-held belief that the driving force of stomatal movement is a turgor mechanism. On illumination the suction pressure of the guard-cells presumably increases and they are thereby enabled to withdraw water from the neighbouring cells; on darkening, the

suction pressure is reduced and the process reversed. We are not here concerned with the more fundamental factors which bring about these changes in suction pressure; for the purpose of this discussion the existence of such changes will be taken as a postulate. Let us now consider the effects of an epidermal water-deficit on such a mechanism.

(i) *Mechanical pressure*

The experiments in which Heath (1938) punctured individual neighbouring cells show clearly that, in *Cyclamen* and *Tradescantia* at least, the turgid neighbouring cells exert an appreciable mechanical pressure on the guard-cells of an open stoma; and there is no reason to suppose that the species in question are exceptional in this respect. Furthermore, the existence of such a pressure provides a plausible explanation of the well-known preliminary opening on wilting shown very clearly in the experiments of Pearse and Spencer; for in the case of such rapid wilting water will be withdrawn from the neighbouring cells before they in their turn can draw it from the guard-cells, and the sudden reduction of the mechanical pressure on the dorsal walls will lead to stomatal opening. A similar explanation, in reverse, would explain the less-familiar phenomenon of stomatal closure following the provision of a water-supply to a wilted leaf. This phenomenon is also shown by an attached leaf when its petiole is cut under water, an effect observed by both Pearse and Spencer and recently reported independently by Saïd and Tolba (1948), who, however, attribute it to a shock mechanism. It must be admitted that Heath (1938) found evidence of shock-closure of this type in *Tradescantia*, but he points out that even in this case an osmotic explanation is possible. A mechanical pressure, or 'tissue-tension effect', may thus be reasonably assumed; and the effect of drying the epidermis will be to reduce it. Now such a pressure, acting on the dorsal walls of the guard-cells, will always favour stomatal closure; its reduction will therefore favour stomatal opening, and the effect of a 'dry' cup should be to *increase* the rate of opening and *decrease* the rate of closing.

(ii) *Suction-pressure gradient*

Superficial consideration would suggest that under a 'dry' cup the neighbouring cells will have a higher suction pressure than normal; and that when the stoma is darkened the suction-pressure gradient presented to the guard-cells will be abnormally steep, causing an *increased* rate of closure. On the other hand, such a heightened suction-pressure gradient will render more difficult the withdrawal of water from the neighbouring cells by an expanding guard-cell, and should result in a *decreased* rate of opening.

Unfortunately the osmotic relationships of the guard-cells are not so straightforward. Let us assume that when the stoma is closed the suction pressure of the guard-cells is equal to that of the neighbouring cells, as it must be if the system is in equilibrium. On illumination, the suction pressure of the guard-cells suddenly rises; water will be withdrawn from the neigh-

bouring cells until the guard-cells once more come into equilibrium with them; water-movement now ceases and the stoma is open. Now, if the postulated epidermal water-deficit has in fact raised the suction pressure of the neighbouring cells, *and if there has been no specific change in the guard-cells*, the latter will now need to draw less water from the neighbouring cells to reach equilibrium; and at equilibrium the guard-cells should be less inflated and the stoma less widely open. In practice, as we have seen, there is no significant difference in aperture; in fact, the stoma appears in both 'wet' and 'dry' conditions to be open to the fullest possible extent. (The claim by Semmens (1947) that under polarized light the guard-cells may become so turgid as to burst seems to the writer to require further evidence before it can be unreservedly accepted.) Four explanations of this puzzling discrepancy are worth brief consideration:

(a) The change in suction pressure induced by 'dry' conditions in the epidermal cells is negligible. This is incompatible with the findings of section (i) above; it is inconceivable that tissue-tension effects of such magnitude could occur in the absence of appreciable changes in wall tension, and therefore in suction pressure.

(b) The guard-cells absorb water-vapour through the ventral wall. If this were the case they would attain a lower suction pressure than that of the neighbouring cells, to which they would in their turn lose water.

(c) The suction pressure of the guard-cells remains positive even when they are fully turgid. Such a state of affairs would render the guard-cells completely independent of suction-pressure changes in the neighbouring cells; but it is meaningless when applied to accepted osmotic systems, and would necessitate the postulation of some 'non-osmotic' water-movement (see, for example, Bennet-Clark, 1948).

(d) The dynamics of the guard-cell wall are such that the cell is stable when the cell is closed or open, but unstable in intermediate positions, the force exerted in regaining stability being sufficient to overcome osmotic forces within wide limits. The possibility of such a system has been previously suggested by the present author (1940) for other reasons, but it seems improbable that it could be reconciled with the experimental results of Heath (1938).

(iii) *The need for a third force*

It is clear that neither of the preceding mechanisms can account for the experimental results. The 'tissue-tension' effect would explain the slight acceleration in opening, but not the marked acceleration of closure; the suction-pressure effect, even in its simplest conception, would apparently do no more than act in the opposite direction. Some force must be found which causes 'dry' guard-cells to move more rapidly in *either* direction. Three possibilities present themselves:

(a) If, as Kisselew (1925) claimed but Linsbauer (1927) denied, stomatal movement is brought about mainly by changes in the permeability of the guard-cell membranes to solutes, it is conceivable that 'dry' guard-cells might

be more sensitive to factors effecting such changes; but the whole conception rests on too slender an experimental basis to be worthy of detailed consideration.

(b) If the 'dry' guard-cell exhibited an increased permeability to CO_2 , movement in both directions would be accelerated (though, it might reasonably be considered, to the same extent) as a result of the greater rapidity of changes in CO_2 -concentration within the guard-cell (Heath, 1948). (I am indebted to Professor F. G. Gregory for this suggestion.)

(c) The sheer dynamics of guard-cell movement, involving as it does considerable changes in shape, may present greater difficulties if the neighbouring cells are turgid.

In our present state of knowledge, no conclusion is possible; the problem must await further work for its elucidation. It is, incidentally, of interest to note that Stålfelt (1929), in his investigations into the effect on stomatal movement of the water-content of the leaf as a whole, observed that light-induced 'photo-active' stomatal movement was at its most active when the water-content of the leaf was slightly reduced (3 per cent. below full turgor in the case of *Vicia faba*). He ascribes this solely to the lowered mechanical pressure of the epidermal cells, stating that only at this 'optimal' water-content are the guard-cells free to execute independent movement in response to changes in illumination; at higher water-contents such movements are modified or completely prevented by tissue-tension ('passive') effects, and at lower water-contents his postulated 'hydro-active' movements come into operation. For the reasons given above, the present author cannot accept the release of epidermal pressure as an adequate explanation of the phenomena observed.

4. Biological significance

If the foregoing discussion is valid, it is natural to consider the possible biological advantages resulting from the provision of a water-barrier at both inner and outer surfaces of the epidermis. Such provision might, for example, act as an additional safeguard against water-loss; in the course of nature, damage to the cuticle—abrasions, cracks, insect-punctures, and the like—must be common, and an inner cuticularized layer would at least provide a second line of defence and give time for the healing of purely superficial wounds. However, this seems quite implausible, and, in view of the striking stomatal effects here recorded, it is surely more likely that the significance, if any, is to be sought in some aspect of stomatal action. If there were no inner barrier, the ordinary cells of the epidermis (being exposed to the saturated internal atmosphere on one side and cuticularized on the other) would be—apart possibly from conditions of excessive drought—at all times fully turgid. We have seen how extraordinarily sluggish stomatal movements become when full epidermal turgor is artificially induced; and how rapidly stomatal movement is effected in a dry atmosphere. Now although it has been customary for some years to regard transpiration as very largely uncontrolled by stomatal aperture, Milthorpe (1948), using an improved technique, has shown that, on the contrary, all but a relatively small proportion of transpiration changes can

be ascribed to changes in stomatal aperture. Transpiration in light is no doubt unavoidable; the stomata of a green plant of normal metabolism must open in the light if the plant is to survive, even though it is thereby exposed to the risk of excessive water-loss. It is therefore not surprising that external humidity has such a relatively small effect on stomatal opening. However, when the light is withdrawn, open stomata in a dry atmosphere become solely a liability; and it is surely not entirely fanciful to see a biological significance in the facts that in a dry atmosphere, in which rapid closure is of value, darkened stomata do in fact close very rapidly; and that in a humid atmosphere, in which closure is unimportant, closure is slow and incomplete. This would, moreover, provide a satisfying explanation of a puzzling but familiar observation: the fact that in hypostomatous leaves the lower stoma-bearing surface has commonly a thinner cuticle than has the upper surface. For were this cuticle to be so thick as virtually to suppress cuticular transpiration entirely, such a form of regulation of the speed of stomatal movement would be impossible.

At this point, despite its pitfalls, the subject of xeromorphy can hardly be evaded. It seems clear that no great significance is to be expected in the disposition of the stomata. In teleological language, a plant of normal metabolism *must* open its stomata for a period in the light, cost what it may in water-loss; every modification which reduces transpiration will also tend to reduce assimilation, and some sort of balance between undesirable extremes must be maintained. In the circumstances, the wide variation in degree of sunkness and protection of the stomata in xeromorphs is not surprising. However, we have already noted that if the water-supply to a leaf is impaired, the stomata can no longer open fully in the light if the external atmosphere is very dry, exhibiting a closing tendency perhaps comparable with Stålfelt's (1929) 'hydro-active' movement but purely epidermal in origin. Is it possible that, for a plant habitually exposed to a dry external atmosphere and an exiguous water-supply, and lacking the advantages conferred by the abnormal metabolism and stomatal rhythm of the succulents, the greatest danger is not stomatal transpiration (which is inevitable), but a degree of *cuticular* transpiration so excessive as to inhibit stomatal opening completely? This suggests an attractive explanation of another puzzling phenomenon which has for long been familiar, the fact that many of the various 'xeromorphic' features of certain leaves—their thick cuticle, their layers of wax, and especially their hairs (cf. the experiments of Sayre (1920) on the function of the hairs on the leaves of *Verbascum thapsus*)—appear to be directed against cuticular rather than stomatal transpiration. In view of the fact that the former is of negligible magnitude compared with the latter, it has been usual to maintain the aesthetically unsatisfactory view that hairs and similar 'protective' devices fulfil no useful function; but if the writer is correct in suggesting that the commonest water-loss danger to which such a leaf is exposed is that resulting from excessive cuticular transpiration, their presence takes on a new significance—to ensure, in defiance of classical tradition, that even in the driest circumstances the stomata can remain *open*.

SUMMARY

1. It is shown that a dry external atmosphere exerts the following effects on stomatal movement:

- (a) A striking acceleration of closure in darkness.
- (b) A slight acceleration of opening in light.
- (c) If the water-supply to the leaf is impaired, an inability to maintain full opening in the light.

Conversely, a saturated external atmosphere induces sluggishness of movement and a tendency to incomplete closure in darkness.

2. These results are considered to support La Rue's contention that the epidermal water-supply is drawn solely by lateral movement from the main veins, and not from the underlying mesophyll. The stomatal phenomena themselves do not appear capable of any simple explanation based on current knowledge of guard-cell physiology.

3. The biological significance of these results is discussed, with particular reference to the problem of xeromorphic structures, for which a new interpretation is suggested.

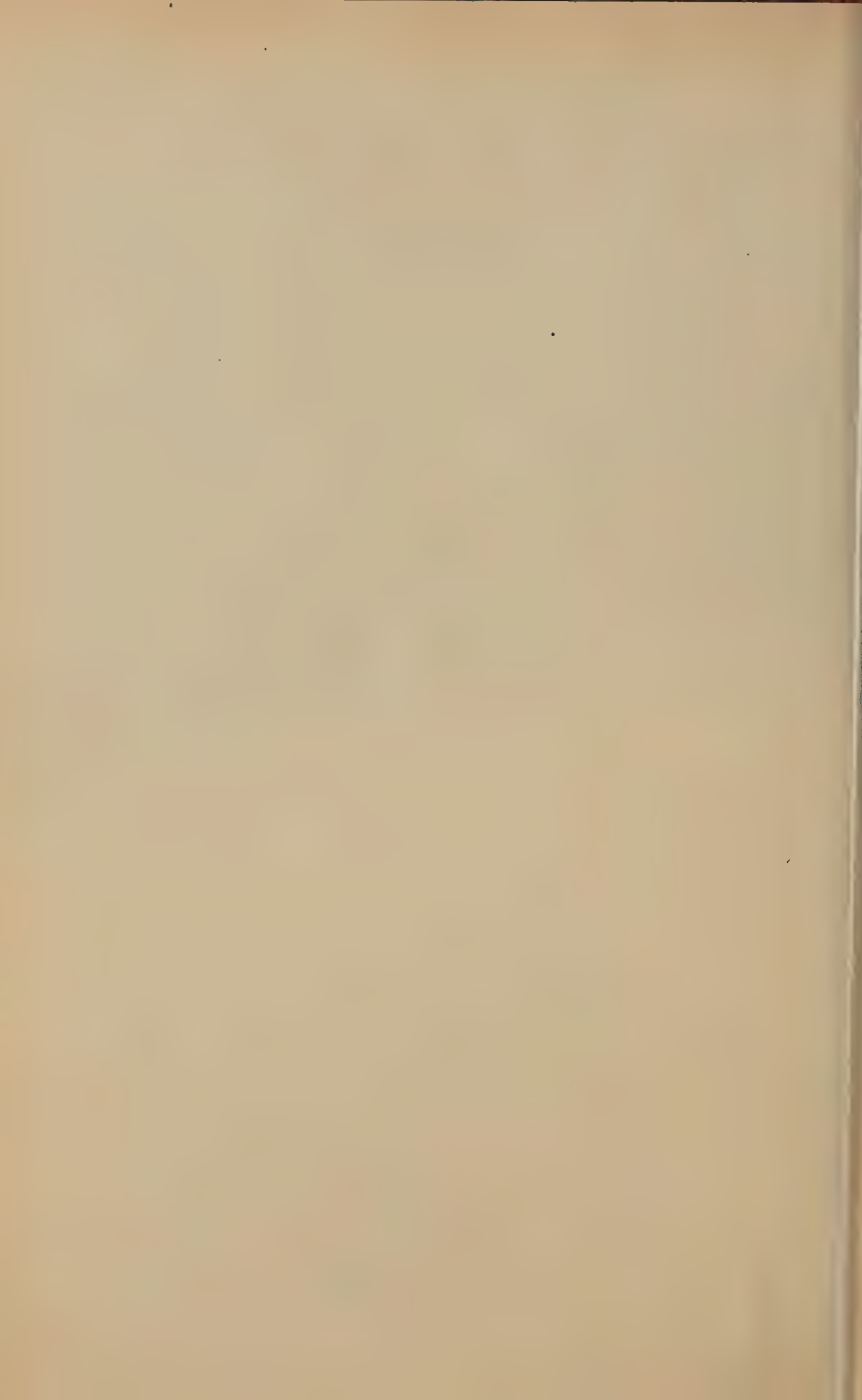
ACKNOWLEDGEMENTS

The writer is especially grateful to Professor D. Thoday, F.R.S., who very kindly made available unpublished data obtained in his laboratories. The substance of this paper was read before the Society for Experimental Biology in January 1949, and among those to whom the writer is indebted for informal discussion it is a pleasure to record in particular his appreciation of the advice and criticism of Dr. O. V. S. Heath. Grateful thanks are also due to Mr. G. Butler, of the Bedford College Botany Garden, who provided the necessary *Pelargonium* plants in very difficult circumstances.

LITERATURE CITED

- AUDUS, L. J. (1939). Mechanical stimulation and respiration in the green leaf. II. Investigations on a number of angiospermic species. *New Phytol.* **38**, 284-8.
- DE BARY, A. (1884). *Comparative anatomy of the vegetative organs of the phanerogams and ferns*. (Translated F. O. Bower and D. H. Scott.) Oxford.
- BENNET-CLARK, T. A. (1948). Non-osmotic water movement in plant cells. *Discussions of the Faraday Society No. 3: Interaction of water and porous materials*, 134-9.
- GREGORY, F. G., and PEARSE, H. L. (1934). The resistance porometer and its application to the study of stomatal movement. *Proc. Roy. Soc., B*, **114**, 477-93.
- HABERLANDT, G. (1914). *Physiological Plant Anatomy*. (Translated from 4th German edn. by M. Drummond.) London.
- HEATH, O. V. S. (1938). An experimental investigation of the mechanism of stomatal movement, with some preliminary observations upon the response of the guard cells to 'shock'. *New Phytol.* **37**, 385-95.
- (1948). Studies in stomatal action—control of stomatal movement by a reduction of the normal carbon dioxide content of the air. *Nature*, **161**, 179-81.
- and WILLIAMS, W. T. (1948). Studies in stomatal action—adequacy of the porometer in the investigation of stomatal aperture. *Ibid.* **161**, 178-9.
- KISSELEW, N. (1925). Veränderung der Durchlässigkeit der Protoplasma der Schliesszellen im Zusammenhange mit stomatären Bewegungen. *Beih. bot. Zbl.* **41**, 287-308.

- LEWIS, F. J. (1948). Water movement in leaves. *Discussions of the Faraday Society* No. 3: Interaction of water and porous materials, 159–62.
- LINSBAUER, K. (1927). Weitere Beobachtungen an Spaltöffnungen. *Planta*, **3**, 527–61.
- MER, C. L. (1940). The factors determining the resistance to the movement of water in the leaf. *Ann. Bot.*, N.S. **4**, 397–401.
- MILTHORPE, F. L. (1948). *Factors in the drought resistance of cereals*. Ph.D. Thesis, University of London.
- PEARSE, H. L. (1935). *The relation of transpiration to stomatal movement and other factors*. Ph.D. Thesis, University of London.
- LA RUE, C. D. (1930). The water supply of the epidermis of leaves. *Pap. Mich. Acad. Sci.* **13**, 131–9.
- SAID, H., and TOLBA, M. K. (1948). The effect on stomatal behaviour of detaching leaves of *Kalanchoë* and *Mesembryanthemum* at different times of day. *New Phytol.* **47**, 284–7.
- SAYRE, J. D. (1920). The relation of hairy leaf coverings to the resistance of leaves to transpiration. *Ohio J. Sci.* **20**, 55–86.
- SCOTT, F. M., SCHROEDER, M., and TURRELL, F. M. (1947). Leaf abscission in the Valencia Orange and suberisation of the internal surface of the Valencia Orange and other leaves. *Amer. J. Bot.* **34** (Abstracts), 589.
- SEMMENS, E. S. (1947). Starch hydrolysis induced by polarized light in stomatal guard cells of living plants. *Plant Physiol.* **22**, 270–8.
- SPENCER, H. J. (1938). *Factors concerned in the water relations of leaves*. Ph.D. Thesis, University of London.
- STÄLFELT, M. G. (1929). Die Abhängigkeit der Spaltöffnungsreaktionen von der Wasserbilanz. *Planta*, **8**, 287–340.
- THODAY, D. (1938). Stomatal movement and epidermal water-content. *Nature*, **141**, 164.
- WILLIAMS, W. T. (1940). *A theoretical and experimental investigation of the factors concerned in stomatal movement, with special reference to the transmission of shock stimuli*. Ph.D. Thesis, University of London.
- (1949). Studies in stomatal behaviour. III. The sensitivity of stomata to mechanical shock. *Ann. Bot.*, N.S. **13**, 309–27.



Studies with Radioactive Tracers in Plant Nutrition

I. PROBLEMS IN THE USE OF RADIOACTIVE TRACERS

R. SCOTT RUSSELL AND R. P. MARTIN

Department of Agriculture, University of Oxford

INTRODUCTION

IN tracer investigations radioactive isotopes are used to show the behaviour of the normal isotopes of the same elements. A small amount of the radioactive isotope (the 'tracer') may be added to the normal isotope (the 'carrier') in the same chemical form, and the 'labelled' mixture is then used in experiments. The movement or utilization of the tracer is observed quantitatively by electronic means or qualitatively by radioautography. The carrier is assumed to behave in an identical manner. Alternatively the tracer may be used 'carrier-free'; here again the interpretation of results depends on the assumption that the tracer behaves similarly to an *equal quantity* of the normal element. Although an extensive literature is available covering both the general aspects of tracer methods (e.g. Kamen (1947), Hevesy (1948)) and their application to the study of plant nutrition, the validity of this assumption in plant studies *during which a significant amount of growth occurs* has received little critical consideration. Likewise many aspects of laboratory procedures have been little defined. A review of the technique from these viewpoints is thus a necessary preliminary to detailed studies of plant nutrition. In the present paper the general validity of the method and its value are considered. Laboratory procedures are described in the second paper of this series.

Throughout the course of any tracer investigation atoms of the radioactive isotope disintegrate. Radiation is emitted and the atom which emits it is usually transmuted into another substance. For example, P^{32} emits an electron and becomes S^{32} . More complex decay schemes frequently occur and some radioactive isotopes, for example Sr^{90} , give rise to radioactive daughters, which in turn decay. The basic principle is, however, the same for all isotopes.

The effects of radioactive isotopes must therefore be assessed from three aspects: (i) their behaviour before the discharge of radiation; (ii) the consequences of the formation of new chemical substances at the instant of breakdown; (iii) the effect of the radiation itself. Only if the behaviour of the tracer has no significant effect in any of these ways can the method yield valid physiological results.

CHEMICAL AND PHYSICAL BEHAVIOUR OF RADIOACTIVE ISOTOPES
PRIOR TO DISINTEGRATION

Since the isotopes of an element differ in mass they cannot be regarded as identical. The isotopes of hydrogen H^1 and H^3 (tritium) differ in mass by a factor of three and the resultant marked differences in their chemical and physical properties limit their use as tracers (Kamen, 1947). Isotopes of heavier elements differ relatively little in these respects and differences in their distribution coefficients are usually insignificant beside other experimental errors (Hevesy, 1948). The recent work of Ogston (1948) and Wilcox (1949) indicates that abnormalities in metabolic process may, however, occur through the process which the latter author describes as 'isotopic pseudo-asymmetry'. In the present work on the absorption of phosphorus by plants no differences in the distribution of tracer and carrier have been noted (except when unsatisfactory sources of P^{32} are employed, see p. 150). This is illustrated by the following figures for the partition of tracer and carrier between roots and shoots of young barley plants, as shown by electronic and colorimetric determinations: The P^{32} used was prepared at the Atomic Energy Research Establishment, Harwell, by the neutron bombardment of sulphur and subsequent oxidation.

Tracer in roots as percentage of tracer in shoots 20.2 ± 2.7

Carrier in roots as percentage of carrier in shoots 21.0 ± 1.8

This agreement, however, does not infer that the movement of a labelled substance will necessarily appear to be the same when determined by electronic and chemical means. If labelled and unlabelled solutions of the same substance are separated by a permeable membrane, normal diffusion will eventually result in the labelled and unlabelled molecules each reaching equal concentrations on both sides of the membrane. Radioactive assay would therefore suggest that an accumulation has occurred on the side of the membrane where the unlabelled substance was originally placed. Chemical analysis would, however, indicate that no accumulation has in fact occurred. Such static equilibria are unlikely in nutrient absorption studies, but ionic exchange can cause comparable effects.

The exchange of potassium between the roots of barley and the culture medium has been investigated by Jenny and Overstreet (1938), Overstreet and Broyer (1940), and Broyer and Overstreet (1940). Some evidence of a similar exchange of the phosphate ion is summarized in Table I. Young barley plants of low phosphorus status were placed in a solution containing 10 p.p.m. labelled phosphorus as KH_2PO_4 . Absorption of tracer and carrier was determined by analyses of the culture solution. The much smaller apparent absorption of carrier in the first day is interpreted as reflecting the loss of phosphate from the plants to the solution. Thereafter no significant difference between the two measures of absorption occurred, indicating that the net rate of absorption of tracer and carrier was now the same. At the end of the experiment the effect of the initial ionic exchange was less than the experimental

TABLE I

Absorption of tracer and carrier by young barley plants treated with 10 p.p.m. labelled phosphorus in water culture.

Time	Absorption expressed as % of initial content of solution		
	Carrier	Tracer	L.S.D.
0-24 hrs. . . .	6.21	9.11	1.02(1%)
24-240 hrs. . .	30.67	29.55	N.S.
Total.	36.68	38.66	N.S.

error. Work on this subject is continuing and will be described later. It is considered here only in so far as it affects the interpretation of results from tracer experiments. If at the beginning of an experiment a plant already contains a nutrient with which it is subsequently treated in labelled form, tracer assay will not necessarily give an estimate of the increased content of the plant. This effect will, however, not be important if the amount of nutrient absorbed is large in relation to the amount of exchangeable nutrient originally present in the plant, and tracer assay should then give a valid measure of absorption. However, when absorption rates are studied over short periods possible exchange effects must be considered in the interpretation of results.

EFFECTS OF SUBSTANCES FORMED BY THE DISINTEGRATION OF ISOTOPES

The decay products of radioactive isotopes come into existence only at the instant when radiation is emitted. They are therefore not measured in tracer assay unless they are themselves radioactive. This, however, does not preclude the possibility that they may cause abnormalities in the experimental organism. For example, P^{32} will enter into the molecular structure of nucleic acid and other organic phosphorus-containing substances. They will be chemically indistinguishable from unlabelled molecules until the P^{32} disintegrates. The phosphorus will then be replaced by sulphur. Neither the biochemical results of this metamorphosis nor its effects on metabolism or chromosome structure have, so far as is known, been studied, though Arnason *et al.* (1948) have considered its possible effects. Since chemical change occurs simultaneously with the discharge of radiation, the difficulty of distinguishing between their consequences is obvious. It is possible that the effects of chemical changes are relatively small and for the present purpose it would seem justified to disregard them.

BIOLOGICAL EFFECTS OF RADIATION

Knowledge of the biological effects of radiation rests mainly on the results of experiments in which external sources of radiation have been used. Many authoritative sources are available (e.g. Lea, 1946; Brit. Radiol. Soc., 1947). α -, β -, γ -, and X-radiation cause ionization in their passage through matter. The depth of penetration and the extent of ionization is predictable for any

type of radiation provided that its energy and the nature of the target material are known. The most serious consequence of such ionizations in living tissues is associated with alterations in the chromosomal structure of meristematic cells. The magnitude of such effects is indicated by the following results of Gray and his co-workers who have studied extensively the effects of ionizing radiation on *Vicia faba* (*Faba vulgaris*) roots (Gray and Read, 1942, 1944; Gray, 1947). Three hours after treatment with 25 roentgens¹ of γ -rays an appreciable reduction in the rate of mitosis is detectable; 100 r. causes a high rate of cell mortality; 50 per cent. of the roots are killed by a dose somewhat exceeding 600 r. Thoday and Read (1947) report that 143 r. may cause 95 per cent. aberrant anaphases after 12 hours in aerated cultures. Considerably less information is available concerning radiation effects which do not result in chromosomal changes. It is, however, of interest that Hevesy (1948) has found that 100 r. depresses the turnover rate of desoxyribonucleic acid. It may be noted also that 0.1 r. per day is the accepted safe dose for man continuously exposed to radiation (Medical Research Council, 1948).

Very low levels of radiation, however, have no significant biological effects. All organisms are, in fact, subject continuously to low though measurable radiation from cosmic rays and naturally occurring radioactive substances. Thus radiation effects in tracer investigations differ in degree rather than in kind from those to which the organisms are habitually subject. If the dosage is kept below the level at which measurable abnormalities occur, no biological significance will consequently attach to the emission of radiation by the tracer.

Such information as is available on cytological and other effects induced by radiation from tracers (e.g. Mullins, 1939; Muir, 1942; Arnason, 1948; Spinks *et al.*, 1948 c) gives no indication that the symptoms differ significantly from those induced by equal intensities of radiation from external sources. The results of experiments in which external sources are employed cannot, however, provide more than a general guide to the situation in tracer experiments. The biological effects of radiation depend both on the total dosage and on the duration of exposure. A short exposure to an intense source may be considerably more damaging than a long exposure to a weak source, although the total dose is the same in both cases (Lea, 1946). In work with external sources the intensity of radiation has usually been considerably higher and the duration of treatment shorter than frequently occurs in tracer experiments. Furthermore, although different types of ionizing radiation have generally similar biological effects, their effects are not identical (Gray and Read, 1944).

The most important difference between the conditions of experiments with external sources and with tracers results, however, from the differences in the distribution of radiation. When external sources are employed the organism

¹ The *roentgen* (r.) is defined as 'that quantity of X- or gamma radiation such that the associated corpuscular emission per 0.001293 gram of air produces in air ions carrying 1 electrostatic unit of quantity of electricity of either sign'. The *equivalent roentgen* (e.r.) is defined as 'that amount of beta radiation which, under equilibrium conditions, releases in one gram of air as much energy as one roentgen of gamma rays'. This unit is also referred to as the *roentgen-equivalent-physical* (r.e.p.) (Marinelli, *et al.*, 1948).

is exposed to a fairly uniform dosage, apart from differences due to the absorption of the radiation by tissues. Different parts of the organism irradiated by an absorbed tracer may, however, receive very different dosages by virtue of the differential distribution of the isotope between different organs. Even if the distribution of the isotope is known, the resultant dose rate cannot be readily determined when the dimensions of the tissue in which it is accumulated are less than the mean range of the radiation. A satisfactory assessment of radiation effects caused by tracers can therefore be made only by direct experiments.

TABLE II

Effect of treatment for 6 days with P^{32} on young barley plants.

Total phosphorus content of culture solution < 0.3 p.p.m.

Concentration of P^{32} in culture solution (initial)	Concentration of P in root tips		Radiation dose e.r./day		Dry weights % of control	
	Concentration of P in culture solution		Culture solution	Root- tips		
					Roots	Shoots
0	—	—	—	—	100	100
0.5	—	—	0.02	—	95	98
10	1,430	—	0.4	300	86*	98
20	730	—	0.8	300	79†	119*
50	300	—	2.0	300	62†	110

* = significantly different from control ($P = 0.05$)

† = " " " " ($P = 0.01$)

(From Russell and Martin, 1949)

It has been shown in this laboratory that when conditions favour nutrient absorption, low activities of P^{32} may cause significant radiation effects on plants both in water culture (Russell and Martin, 1949) and in soil (Russell, Adams, and Martin, 1949). 10 microcuries¹ P^{32} per litre of culture solution has caused a significant reduction in root growth (Table II). The radiation dose in such solutions is 0.4 e.r./day, but owing to the accumulation of phosphorus in root-tips these organs received 300 e.r./day. The pattern of radiation damage is more complex in the soil on account of the presence of an alternative phosphorus source in the soil phosphorus. The most readily discernible effect of radiation damage in this case has been an alteration in the ratio of fertilizer to soil phosphorus absorbed (Table III). This is believed to be explained by the fact that the bulk of the fertilizer is retained in the upper 2 in. of soil and that radiation effects in this region result in a relatively greater absorption by roots in the lower zones. Changes in the partition of phosphorus between roots and shoots have also been observed. The extent of such effects is variable, being related to the rate of phosphorus absorption of the plants. These results

¹ The curie (C) is defined as 'that quantity of any radioactive element that disintegrates at the rate of 3.7×10^{10} atoms per second'.

0.001 curie = 1 millicurie (mC)

0.001 millicurie = 1 microcurie (μ C)

1 microcurie of P^{32} weighs 3.5×10^{-12} g. (Evans, 1947).

TABLE III

Effect of varying activities of P³² on growth of barley

All treatments received the equivalent of 8 lb. total phosphorus/acre. Harvested 39 days after treatment.

	Treatment: $\mu\text{C P}^{32}/\text{g. P}$			
	0	18	92	460
Dry weight Shoots (g./pot)	2.38	2.46	2.51	2.63
" " Roots "	1.23	1.22	1.26	1.17
Fertilizer P absorbed (mg./pot)	—	3.42	2.75	2.45*
Fertilizer P absorbed $\times$ 100/Soil P absorbed	—	16.74	12.4*	7.9†

* = significantly different from control ($P = 0.05$)

† = " " " " " ($P = 0.01$)

(From Russell, Adams, and Martin, 1949)

indicate that radiation effects in nutritional experiments merit greater consideration than they have received from earlier workers in this field (Dean *et al.*, 1947; Spinks *et al.*, 1947, 1948 *a, b*) who have frequently used considerably higher activities of P³². It is to be noted that the appearance and weight of shoots (Tables II and III) may give no indication of radiation effects even if their extent is considerable. Some of the results suggest an increase in shoot growth, presumably as a result of interference with downward translocation. These facts provide some explanation of the conclusions of Hendricks and Dean (1947) and Dion *et al.* (1949) who rejected the possibility of radiation effects in nutritional studies without examining root systems. This subject will be considered more fully in a subsequent paper in this series.

So far as is known, comparable studies with other isotopes have not been undertaken, but it may be expected that elements such as potassium, which are also accumulated in meristems, will show similar effects. Plants must therefore be regarded as particularly sensitive to radiation damage from isotopes which are accumulated metabolically. In the animal body the situation with regard to phosphorus is very different; the extent of accumulation is comparatively small and the rate of elimination is rapid. Marinelli *et al.* (1948) found that in consequence of this no tissues in mice treated with P³² received the theoretical dose calculated from the concentration of isotopes applied and the weight of the body. P³² is, in fact, regarded as one of the safest isotopes from the viewpoint of animal hazards. The accumulation of certain other isotopes in animals, however, provides a parallel to the hazard of P³² in plants. Iodine, for example, is accumulated in thyroid; strontium, and many other elements in bone; radioactive isotopes of these elements are therefore damaging to animals in quantities much below the tolerance level for P³² (Medical Research Council, 1948).

From the foregoing it is clear that the biological effects of radiation from tracers are complex and variable. All factors which influence the rate of absorption of the tracer will interact in determining the resultant radiation dose in the tissues. The metabolic role of the labelled substance, its con-

centration, and the metabolic activity of the plant are of particular importance in this respect. A reduction in the level of carrier, other conditions remaining unchanged, is likely to result in an enhanced level of radiation in those tissues where the tracer is accumulating.

An essential preliminary to tracer work must be the experimental determination of the tolerance level, by varying the amount of tracer under the chosen experimental conditions and observing if the rate of the process under investigation is affected.

The importance of avoiding radiation effects will vary with the nature of the investigation. Since the rate of nutrient absorption can be greatly affected by radiation, it is patent that the avoidance of radiation effects is highly important in quantitative nutritional experiments. Qualitative studies of the path of translocation or mode of utilization of substances may, on the other hand, yield valid results when mild degrees of radiation damage occur provided that the rate, but not the form, of growth is affected. In experiments of very short duration difficulties from this cause should be less likely to occur. At the present time there is insufficient data to indicate the extent to which radiation effects limit the scope of tracer investigations. It is, however, evident that work of many types can be safely undertaken if tracer levels are carefully controlled.

In the light of the above considerations, it appears that the behaviour of radioactive isotopes, both before and at the time of their disintegration, is such that, if conditions are carefully controlled, tracer methods can yield valid results in biological studies. At the same time it is apparent that considerable and carefully planned safeguards are necessary if misleading results are to be avoided, especially when the experimental material is of high metabolic activity.

THE VALUE OF TRACER METHODS

The types of observation for which radioactive tracers are particularly valuable can be classified under four main headings: the estimation of very small quantities of substances; the labelling of one fraction of a substance to distinguish it from unlabelled fractions of the same material; *in vivo* determinations of the movement of substances; the location of materials in tissues by radioautography.

Emissions from individual atoms are detected by tracer assay methods, and although only a small proportion of the radioactive atoms in a sample emit radiation during an assay, the method is infinitely more sensitive than the most accurate chemical and spectroscopic tests, which detect substances only when the number of molecules present is very large—usually in excess of 10^{16} . The smallest amount of carrier-free P^{32} which can be conveniently measured by the equipment used in this laboratory is 10^{-15} g. The addition of carrier decreases the sensitivity of the method, but the lowest ratios of tracer to carrier which it has been found desirable to use for many types of work allow quantities of less than 10^{-8} g. to be measured accurately. The great

advantage of this sensitivity in assaying microscopic samples or in studying the distribution of substances present in very low concentrations is clear.

Radioactive assays are unaffected by the presence of non-radioactive materials, except in so far as they absorb radiation, and for this allowance can be made. Thus a labelled fraction can be distinguished from unlabelled fractions of the same substance. On this depend the widest possibilities of tracer work. Examples in different fields are Calvin's studies of the intermediary substances in photosynthesis (*vide* Benson and Calvin, 1948), the work on cationic exchange already cited, and numerous investigations of nutrient utilization (e.g. Arnon *et al.*, 1940; Biddulph, 1948; Broyer and Hoagland, 1943).

In vivo determinations can be carried out by placing a counter close to a living plant to which a tracer has been administered. Although the absorption of radiation by the tissue and the variable shape of the specimen frequently make quantitative determinations difficult, this technique is none the less of obvious potential value for observing accumulation rates by consecutive observations on a single individual. Similarly, in water-culture studies it is possible to record the changing concentration of culture solution. Appropriate laboratory procedures for these applications have as yet been little developed.

The sensitivity of photographic emulsions to radiation enables radioautographs to be prepared showing the distribution of tracers within tissues. Since radiation cannot be focused, high resolution depends on thin sections being placed in close contact with the photographic emulsions (Pelc, 1947; Yagoda, 1949; Russell, Sanders, and Bishop, 1949). Points 15μ apart can be readily distinguished, while in favourable circumstances the position of materials within individual cells has been indicated.

If observations of any of the above types are essential in an investigation, the value of tracers may be considerable, provided that a suitable isotope is available. However, it does not follow that tracer methods will necessarily yield new information. If the sensitivity and accuracy of chemical methods of analysis is adequate, the greater sensitivity of tracers will be without value. Again, if it is desired to show the distribution of substances between tissues which can be easily dissected apart and separately analysed, radioautographs are without special advantage. When more than one experimental procedure is available, the choice of method must clearly depend on the relative convenience, reliability, and cost, both in time and money, of the alternative procedures. These practical aspects of tracer work are discussed in the paper which follows.

Studies with Radioactive Tracers in Plant Nutrition

II. THE ESTIMATION OF RADIOACTIVE TRACERS

R. P. MARTIN AND R. SCOTT RUSSELL

Department of Agriculture, University of Oxford

INTRODUCTION

Two types of procedure are used to identify radioactive tracers—electronic assay and radioautography. In this paper consideration will be given only to assay methods, and in particular to those which have been found suitable for estimating labelled phosphorus in plant material. The equipment here used consists of a Geiger Muller counter tube supplied with an appropriate voltage and connected to a recording unit. This may take the form of an electronic scaling unit which records the number of pulses generated in the counter or a rate of count meter which measures the mean rate of emission. The former method is the more accurate and is therefore preferred for assay purposes. The physical principles of the method have been discussed by Lewis (1942), Korff (1946), and other authorities; they are considered here only in so far as is necessary for the discussion of the suitability of different types of equipment and for the assessment of experimental errors in the present work.

ELECTRONIC EQUIPMENT

Geiger Muller Counter Tubes

Two types of counter have been found convenient for assay purposes:

(a) A bell-shaped counter with an end window of copper foil 20 mg./cm.² in thickness (Type G.M. 2, made by General Electric Co. Ltd.). This is suitable for assaying small solid samples which are usually mounted on metal disks and placed on a small tray below the window. Liquid samples of high activity can also be assayed, but greater sensitivity is obtained with the glass counter described below. (An improved type, G.M. 2A, with a dural window of equivalent thickness, has recently been developed to replace the G.M. 2.)

(b) A thin-walled (25 mg./cm.²) cylindrical glass counter surrounded by a jacket into which a sample of liquid 10 ml. in volume can be placed (type M.6, 20th Century Electronics). This counter, which was designed by Veall (1948), will record up to 10 per cent. of the emissions from a liquid sample containing P³². This compares with 25 per cent. of emissions recorded when small samples are placed close to an end window counter. This smaller sensitivity is offset for many types of work by the greater convenience and accuracy with which liquid samples can be handled. Moreover, greater reproducibility is attained. Small variations in position cause considerable variations in the proportion of particles when small samples are placed close to end window counters. No such variations can occur in the introduction of

liquid samples into the glass jacket. M.6 counters are sensitive, in varying degrees, to photons. It is therefore important to shield them from light.

The operation of a counter is dependent upon the voltage supplied. Below a certain voltage, the 'threshold', the counter will not operate, and above a certain maximum, large numbers of spurious pulses occur, until at very high

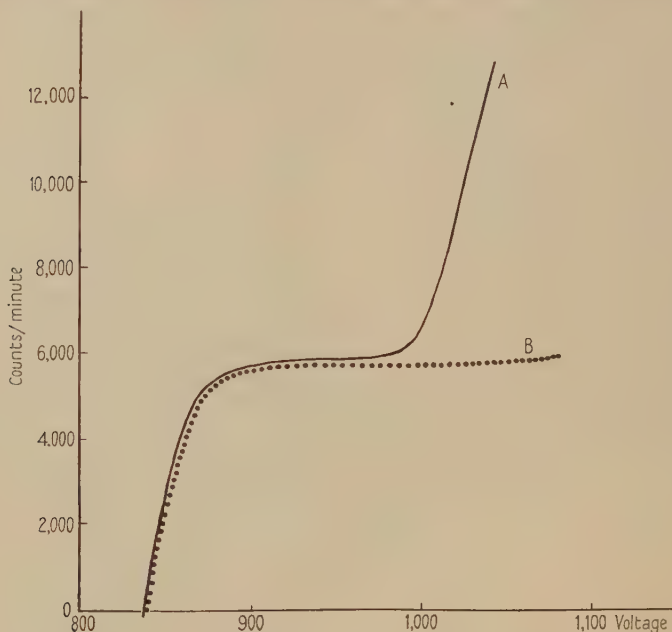


FIG. 1. Effect of voltage on counting rate of an M.6 liquid counting tube.
A = without quenching circuit; B = with quenching circuit.

voltages the tube discharges continuously. Over an intermediate range, the 'plateau', the counting rate is more constant and almost every particle which enters generates a pulse. During the operation of a counter the voltage is maintained near the centre of the 'plateau'. Between counters, even if they are of the same type, considerable differences occur in the extent of the plateau, its voltage, and its slope. It is desirable for the plateau to extend for at least 200 volts. A cause of slope in the plateau is the production of more than one pulse by a single emission. The effect is reduced in the counter tubes here used by the inclusion of an organic quenching vapour in the gas mixture with which they are filled. An electronic quenching circuit designed by Cooke-Yarborough *et al.* (1949) (manufactured by Dynatron Radio Ltd., type 1014 A) is also used. This reduces the voltage after each pulse to a level below the plateau for a constant period which exceeds the normal 'dead time' of the counter. The effect of this circuit on the plateau of an M.6 counter is shown in Fig. 1. The subject is treated fully by Putman (1949).

The quenching circuit serves the further purpose of making the resolving time of the counter constant, whereas the dead time of a normal counter is subject to variation. The greater precision with which a statistical correction for unrecorded counts can be made offsets the resultant increase in resolving time. In practice the resolving time of the system is adjusted to 300 microseconds. The percentage of unrecorded counts (C) is then calculated by the formula $C = N/1800$, when N is the number of counts observed per minute, and this resolving time correction is added to the observed counts. As the error of the correction increases when it exceeds 10 per cent., the counting rate of samples is kept below 20,000 counts per minute.

When no sample of radioactive material is presented to the counter, stray radiation causes a slow background counting rate. Especially when counting rates are low, a high background greatly reduces the precision of an assay. Counters are therefore enclosed in 'lead castles' 2 to 3 in. thick, which shield them from all but the most penetrating cosmic rays. This reduces the background of the counters to 8–10 per minute, approximately one-third of the rate for an unshielded counter. In practice, the lead castle serves the secondary function of providing a stand for the counter and for the sample, if it has to be supported externally.

High-voltage power supply

The variations in the efficiency of a counter which result from changing voltage make a stabilized power supply essential. Furthermore, the output voltage must be adjustable so that each counter shall be supplied with the correct operating voltage. Two types of unit developed by the A.E.R.E., Harwell, and manufactured by Dynatron Radio Ltd. are used. One is a complete unit (Type 200) to supply a single counter; the other (Type 1007) provides for the operation of a number of counters. A common power unit generates a fixed voltage from which each counter is fed through a separate calibrated potentiometer. If two or more counters are situated close together, the second type has advantages both in cost and in size.

Scaling units

The scaling unit is not only one of the most complicated items of equipment required for tracer work but also one of the most expensive, and it is more susceptible to faults than any other item of equipment. The choice of a satisfactory type is therefore a matter of high importance. The complexity of the apparatus is largely dependent on the maximum counting rate it is necessary to record, and since both cost and liability to faults increase with complexity, the desirability of employing the simplest adequate apparatus is apparent. Experience in this laboratory has indicated that little advantage results from the use of a scaling unit capable of recording counts in excess of 6,000–10,000 per minute. Much lower counting rates are usual and more active samples can readily be diluted if the M.6 liquid counter is used. With an end window counter the position of the sample can be altered or an absorber introduced.

The most satisfactory scaling units available in Britain have in the main been developed for nuclear physical research (e.g. Dynatron Type 200) and are capable of recording counting rates up to 50,000 per minute. They are, therefore, unnecessarily complicated for the work and it is relevant to review the principles of scaler design to indicate both the disadvantages of present equipment and possibilities of improvement.

Electronic scaling circuits are employed because mechanical registers are usually unable to operate sufficiently rapidly to record the pulses generated in the counter unless the counting rate is very small.

The Eccles-Jordan 'scale-of-two' is one of the simplest scaling devices. Two valves are interconnected in a symmetrical circuit which is capable of two stable states depending on which valve is conducting. When a pulse is fed in the circuit changes over from one state to the other and each valve conducts once for every two pulses received. Thus by feeding pulses through the circuit the pulse-rate can be reduced by a factor of two. The process can be continued further by connecting a number (x) of such stages in cascade to give a factor of 2^x . One unit recorded on a mechanical register operated by this system will therefore represent 2^x pulses. At any time a number of pulses varying from zero to $2^x - 1$ will be represented by the states of the stages in the cascade. This can be determined by indicator lamps fitted to each stage. The lamp connected to the first stage will represent 1, and subsequent ones 2, 2^2 , 2^3 , . . . Not only the pulse-rate but also the variance in the time interval between pulses is reduced by scaling and the counting rate which can be recorded by the mechanical register is increased by a factor considerably in excess of the scale factor.

This form of scaling unit has the disadvantage that the readings on the register must be multiplied by 2^x to obtain the number of counts. This is obviated in a modified circuit designed by Rotblat *et al.* (1948). Four scales-of-two are so connected that, after nine pulses have been recorded in the manner described, the tenth pulse returns all the stages to their original states and feeds one pulse to the next set of stages. Thus scale factors of 10, 100, &c., are obtained.

A defect in many circuits, including that used in the Dynatron scaling unit Type 200, is that if one valve or associated component fails the stage of which it is a part may be by-passed and the pulse fed straight to the next stage. The scale factor will therefore be reduced. A fault of this type can readily pass unnoticed, especially if it is intermittent, which happens not infrequently. Furthermore the complicated scale-of-ten circuits are more liable to faults than the simpler scales-of-two.

In the light of these considerations an experimental scaling unit of simple design has been constructed, comprising three scales-of-two (scale factor 8) and a high-speed recorder capable of accepting 50 counts per second. A counting rate of 6,000 per minute is attained. Modifications of the scale-of-two circuits to give the maximum reliability and to prevent an inoperative scaling stage being by-passed are being explored. Alternative mechanical

registers are also being examined. When the reliability of the apparatus has been sufficiently tested in routine use it will be described.

Timing units

When the activity of samples is low, counting times of 20 minutes or longer are frequently necessary to obtain statistical accuracy. In such circumstances an automatic timing unit is of great value as the operator is able to proceed with other work or to use more than one counter simultaneously. The unit may either operate the scaling unit for a given period and record the total number of counts or record the time for a given number of counts. Such timing units are available for use with the Dynatron Scaling Units, but they are unnecessarily complicated for routine work of the type involved in nutritional studies. A simpler apparatus which will operate only for a number of fixed times has been built in this laboratory at small cost and has proved of great value. It consists of a clockwork-driven contactor which makes a contact every half-second. Impulses from this are used to drive uniselector mechanisms to give the desired time intervals which in this case are 2.5, 5, 10, and 20 minutes. A number of scaling units can be controlled by uniselectors driven by a single contactor. Great variation is possible in the design of such an instrument depending on the components available.

Radiation monitors

Monitors are used to detect the general level of radiation in the laboratory for the purpose of reducing experimental errors and safeguarding the health of workers (see Medical Research Council, 1948). For the detection of contamination from β -radiation sources, a monitor comprising a cylindrical glass counter (Type B 12 manufactured by 20th Century Electronics,) and connected to a rate of count meter of conventional design has proved satisfactory. A dose-rate meter incorporating an ionization chamber is used when γ and high-activity β -rays are present.

LABORATORY PROCEDURES

For the assaying of plant material, liquid counting with the M.6 tube has been found more satisfactory than the assay of dry samples with an end window counter. Not only is greater geometric reproducibility attained in the counter but also the preparation of samples is simpler and losses are negligible by comparison with those involved in the manipulation of small samples of dry ash or precipitate which would frequently be less than 50 mg. Hitherto dry samples have usually been used in the assay of P^{32} in nutritional studies (Dean *et al.*, 1947; Nelson *et al.*, 1947; Spinks *et al.*, 1947, 1948*a, b*). Hall and MacKenzie (1947) report unfavourably on liquid counting procedures both because of the greater complexity of laboratory operations and the adsorption of P^{32} on glass walls. Manipulative difficulties have been largely overcome by the convenient design of the M. 6 counter, while adsorption can be prevented

by adjusting the acidity of the sample to pH₃ or less. It is found that two washings with distilled water reduce the background of the M.6 counter to normal after the assay of such samples.

Sample preparation for determination of total phosphorus content

(i) *Plant tissues.* The method of wet ashing is varied according to whether or not a chemical estimation of total phosphorus is required. If so, the following procedure is adopted and the total phosphorus is determined by the method of Allen (1940). Up to 2.5 gm. of dried plant material is placed in a 50-ml. Kjeldahl flask. 2 ml. of 60 per cent. perchloric acid (A.R.) is added, followed by nitric acid (A.R.), the quantity of which depends on the nature of the material. With young barley roots or shoots a quantity sufficient to damp the material has proved satisfactory, but more acid is necessary for the treatment of the roots and leaves of bean plants. When insufficient nitric acid is used a colourless residue is not obtained, but it is desirable to use the minimum adequate quantity since all the nitric acid must be driven off before the completion of the process.

If colorimetric determinations are not required, the cost of reagents is reduced by using concentrated sulphuric acid ('pure') in place of perchloric acid and 'pure' instead of A.R. nitric acid. Otherwise the procedure is unchanged.

The Kjeldahl flasks are heated cautiously on an electric hot-plate. With large samples a vigorous reaction occurs after a few minutes, and heating is stopped until it has subsided. The flasks are then heated until all the nitric acid has been removed and they contain a liquid which is either colourless or a pale yellow which becomes colourless on cooling. If necessary further nitric acid is added and the tubes are reheated. The time for the complete process varies from $\frac{1}{2}$ hour to 5 hours according to the size and nature of the sample. The explosive nature of mixtures of perchloric acid and organic matter has caused difficulties only when large samples are treated in 250-ml. flasks, presumably on account of local heating. All flasks are, however, shielded by safety-glass screens.

It has been found convenient to construct long narrow hot-plates for heating the flasks. Each accommodates twelve flasks resting in shallow depressions on the plate, which is made of aluminium and heated by a coiled element (1,000 watt) approximately 1 in. below. This equipment is simple and inexpensive to construct.

(ii) *Very small plant samples (for radioactive assay only).* Samples of such dimensions that they can be mounted on a nickel disk 1.5 cm. in diameter can be counted intact with a G.M. 2 counter. Root-tips and other pieces of tissue up to 5 mm. long and not more than 1 mm. thick can be conveniently handled in this way. Since such tissues are too small to be weighed accurately on a chemical balance, their volumes are determined under the microscope. If placed wet on to a disk, they normally stick closely to the surface on drying. Any tendency to curl can be overcome by smearing the disk with a film of adhesive.

Normally three to five root-tips are a convenient sample. Errors due to absorption of radiation by tissues and to the non-uniformity of their position clearly occur, but are small in the work for which this method has so far been used. The technique could, if necessary, be elaborated to give more accurate results.

(iii) *Culture solutions.* Samples of culture solutions are acidified to pH 3 or less immediately they have been taken to prevent both losses during storage and the contamination of the counter tubes. When the total phosphorus content of the solution is much less than 1 p.p.m. a further safeguard against losses by adsorption is to increase the phosphorus content to this level by the addition of carrier. This precaution is, however, only necessary if the samples are stored for several days. Samples of culture solutions treated in this way have given correct results when assayed after varying periods.

Should the culture solution have been in use for so long that a significant bacterial population has developed, contamination of the counter may arise from the organisms adhering to its walls. Filtration is necessary in such circumstances.

Sample preparation for other purposes

The wet ashing method is applicable to substances other than phosphorus, provided they are not volatile or do not yield volatile products in the ashing process. It is impossible to prepare samples for assay of labelled iodides in this way since iodine is given off. Heating with a strongly alkaline iodide solution and subsequent filtration gives satisfactory results, but is not convenient owing to the gelatinous nature of the material after heating. Extraction is rendered simpler by macerating the tissue and using a lower concentration of alkali.

A promising extraction method for the determination of the water-soluble fractions of labelled substances is to place the material in a finely divided state in a solution of the unlabelled substance and then allow the material and solution to reach equilibrium. The liquid is then filtered off and an aliquot counted. This method has been used by Aronoff and Calvin (1948).

Calibration of counters

Since the amount of labelled substance in a sample can be determined by comparing its activity with that of an aliquot of the original preparation, it is not essential in all tracer work to know the activity of tracers in absolute units. This simple procedure is, however, unsatisfactory in nutritional studies, since injuries due to radiation can occur unless the level of tracer is carefully controlled. The difficulty of determining activities in absolute units is illustrated by the fact that until recently the standards for P^{32} used in different major institutions differed by a factor of two. Calibration sufficiently accurate for the present purpose is, however, possible by counting a standardized sample and calculating a 'counter factor' which when multiplied by the number of counts recorded in a given time gives the activity of the sample in microcuries. The standards used are obtained from the A.E.R.E., Harwell.

Each counter tube is calibrated separately for each isotope. The

necessity for this is illustrated by the following counting rates of different liquid counter tubes (M.6) for one sample of P^{32} and for one of I^{131} . These two tubes were the extremes in a batch which was tested.

Tube . . .	1	2
P^{32} . . .	2340	2260 counts/min.
I^{131} . . .	4110	2330 „ „

It will be seen that not only do the tubes differ in efficiency but that the extent of difference is not constant for the two isotopes. This is largely due to differences of window thickness which have the greatest effect on the penetration of radiation when it is of low energy. The maximum energies of the radiation from P^{32} and I^{131} are 1.69 and 0.60 MeV respectively. I^{131} emits γ as well as β radiation, and differences in γ -ray efficiency may contribute to the observed differences in counter efficiency.

A well-constructed counter will maintain a constant efficiency over a considerable period if the operating conditions are kept constant. Changes in efficiency may, however, occur from ageing, misuse, or constructional defects. Counters are therefore checked at regular intervals. On account of radioactive decay, standard samples of P^{32} and other isotopes of relatively short half-life cannot be kept for considerable periods. The frequent preparation of new standards is obviated by employing secondary standards of long half-life. For this purpose uranium salts have been used. For end window counters (G.M. 2) the material is mounted on a counting disk and coated with a protective layer. When solutions of uranium salts are used in M.6 counters, difficulties have resulted from contamination. A useful standard for such counters has, however, been prepared by coating with a uranium salt the outer surface of a thin glass tube which will slide into the counter. The uranium layer is protected with 'Scotch tape'. Despite unavoidable variations in the thickness of the uranium layer and in the position of the tube when it is inserted into the counter, this standard gives adequate reproducibility. Solutions of salts of Co^{60} (half-life 5.3 years) are also used to check liquid counters, but the uranium standard has been found more convenient, both because no correction for decay is necessary, and because there is no need to wash the counter after the standard has been used. Since these secondary standards do not emit radiation of the same type and energy as the isotopes to be assayed, they are used only to determine whether a counter has changed in efficiency. New counters are calibrated with samples of the isotope in question which have been standardized on a counter of known efficiency.

Counting routine

The general procedure for carrying out assays is indicated by the foregoing descriptions of the apparatus and the methods for sample preparation and counter calibration. The operating voltage of the counter is carefully adjusted each time the apparatus is brought into use. The threshold voltage is checked, and if it is correct the power supply is set to the predetermined operating

voltage of the counter. If the threshold is incorrect, the nature of the fault, which may be due to the counter or to the power supply, is investigated.

Before the first sample is introduced into the counter the background is counted. If it is significantly above the expected value the cause is investigated. Probable causes of a high background are the presence of a radioactive contaminant in the counter or an excessive operating voltage. Voltages above the plateau may damage the counter temporarily or permanently. Thus if a fault in the power pack is suspected, the counter is not operated until the fault has been investigated. When the background counting rate has been shown to be satisfactory, a standard is counted to check the calibration of the counter. Assaying is then commenced.

In counting liquid samples with the M.6 counter two tubes are used alternately. One is emptied, washed, and filled, while the sample in the other is being counted. The voltage is adjusted before the commencement of each count if the operating voltages of the two counters are not identical. This procedure would be liable to cause errors if the power source were not fitted with a sensitive and reliable voltage control, but with the power units here described, no such difficulties are experienced.

The maximum counting rate of samples assayed with the M.6 counter fitted with a quenching circuit and connected to a Dynatron 200 Scaler is kept below the arbitrary limit of 20,000 counts/minute. Above this rate errors in the resolving time correction rise steeply and the likelihood of difficulties due to contamination and other causes is increased. More active samples are therefore diluted.

After a sample has been counted the counter is washed first with distilled water and then with acetone, drained, and filled with the next sample. It is important to dry the outside of the counter as moisture may cause an electrical leak across the mercury contacts at the bottom of the lead castle. It may be noted that the construction of the counter is such that if a sample slightly in excess of the standard volume (10 ml.) is inserted, no appreciable alteration in the counting rate occurs in the assaying of P^{32} . If the tracer emits γ -rays, differences may, however, occur. At regular intervals the background is checked to ensure both that the counter is working correctly and that no contamination has occurred.

When many liquid samples of variable activity are to be assayed it is found convenient to make a preliminary 'rough count' of the samples in the tubes in which they are stored. A counter unshielded by a lead castle and connected to a scaling unit or a monitor is adequate for this purpose. The weakest samples are selected and assayed first, and the extent of dilution necessary for highly active samples is indicated.

Computation of results

The activity of a sample in μC is obtained by multiplying the observed background, corrected for resolving time, background, and if necessary self-absorption (see p. 152) by the counter factor. If a significant amount of radio-

active decay has occurred during the experiment, the activity is corrected to a chosen reference time. Since the decay of isotopes is exponential, tables for the correction are readily prepared if the half-life of the isotope is known. The amount of labelled substance in the sample is obtained by multiplying the corrected activity by the weight of labelled substance which contained an activity of $1 \mu\text{C}$ at the reference time.

SOURCES OF ERROR

It has been found that the following sources of error merit attention. The systematic nature of several of the errors precludes their statistical assessment, and the obtaining of valid results therefore depends on their control.

(i) *Unsatisfactory tracer preparations.* It is obviously essential that any radioactive isotope used as a tracer shall be of the same chemical form as the carrier. Unfortunately the most simple method of preparing labelled substances—the irradiation of salts in the uranium pile—is for this reason sometimes unsatisfactory. Libby (1940) has shown that, when a phosphate is irradiated, up to 40 per cent. of the P^{32} may be in the form of phosphite. Such preparations are clearly unsatisfactory for tracer studies. The effect of using P^{32} prepared in this way is shown by the following figures for the partition of tracer and carrier between roots and shoots of young barley plants as shown by electronic and colorimetric determinations of phosphate in plants that had been treated with labelled phosphorus for 6 days:

Tracer in roots as percentage of tracer in shoots	24.1 ± 3.5
Carrier in roots as percentage of carrier in shoots	11.1 ± 4.4

The discrepancy was apparently due to the low mobility of the phosphite ion. When the tracer was prepared by the oxidation of P^{32} derived from the irradiation of sulphur, the two methods of analysis were in agreement (see p. 134). Evidence on the behaviour of irradiated phosphate has also been obtained by Thomas and Nicholas (1949). It is apparent that unless they are thoroughly oxidized after irradiation such preparations are likely to cause erroneous results. Some investigators (e.g. Dean *et al.*, 1947), however, have used irradiated phosphate, but it is not clear from the literature whether effects of the above kind were considered. Little information is available as to the possibility of similar errors arising with other isotopes.

(ii) *Contamination.* Owing to the minute quantities of radioactive tracer which are detectable (see p. 147) the contamination of glassware, bench surfaces, and the hands of workers can lead to much greater errors than in chemical analysis. The probable extent of contamination depends on the nature of the tracer used. With P^{32} a special problem arises due to adsorption. It is discussed in the section which follows. With volatile tracers such as I^{131} the danger of contamination is also great.

Extreme care in the handling of all active solutions is therefore essential, and also scrupulous cleanliness akin to that of a bacteriological laboratory. In addition to reducing experimental errors such precautions are an important safeguard against injury to the health of workers.

(iii) *Losses due to adsorption.* In water-culture experiments the adsorption of phosphate from solutions on to the walls of culture vessels presents a serious problem, especially when low concentrations of phosphate (i.e. down to 0.001 p.p.m.) are employed. It cannot be eliminated by the addition of acid as is possible in liquid samples.

TABLE I

Adsorption of phosphate on walls of 300-ml. glass culture jars from culture solution containing 0.15 p.p.m. P (pH 6)

Time (days)	% absorbed	
	Untreated jars	Jars coated with silicone
1	3	5
2	21	21
5	77	80
6	83	85
17	97	97

The extent of adsorption loss may be affected not only by the concentration of phosphate but also by the duration of the experiment, the pH of the medium, the nature and condition of the surface of the containing vessel, and its surface area per unit volume of solution. The extent to which adsorption can occur is shown in Table I. In the greater part of the present work the pH of culture solutions is adjusted to pH 6. No significant errors attributable to adsorption are observed when the concentration of total phosphate is 1 m.eq./l. or higher. When lower concentrations are used it is, however, frequently necessary to take adsorption into account in the assessment of results. The extent of the losses resultant from this cause is determined in such cases by drawing up a 'balance-sheet' for each culture or by subsequently releasing the adsorbed phosphate with acid and assaying it. The possibility that adsorbed phosphate may be released into the solution as a result of falling concentration during experiments must be envisaged. Under the present experimental conditions this effect, however, appears to be small. When culture jars on to which labelled phosphate has been adsorbed are filled with a phosphate-free culture solution, no significant release of adsorbed phosphate is detected over periods of a week—the longest period for which plants are normally left in any solution. Thus in some types of work it may be possible to reduce adsorption by filling vessels with culture solution and allowing them to stand prior to the commencement of an experiment. The more direct method of preventing adsorption by lining the vessels with a suitable material merits consideration. In this laboratory a proprietary silicone (Table I) and methyl methacrylate have proved ineffective for this purpose. Hall and MacKenzie (1947) have found butyl methacrylate of value, but it appears that they employed higher phosphate concentrations than those frequently used in the present work.

(iv) *Instrumental errors.* The considerable errors which can result from the failure of components or the incorrect adjustment of the operating voltages of

counters have been indicated. The seriousness of such effects is increased by that fact that in the absence of careful supervision they can readily pass undetected and, when suspected, decay of the sample may preclude repetition of the analysis.

Further instrumental errors are caused by temperature changes. The rate of disintegration of isotopes is independent of temperature, but at low temperatures the reduction in pressure of the quenching vapour in the counters results in inadequate quenching. Thus spurious pulses are caused. This effect has been observed at temperatures below 15°C ., and the laboratory temperature is therefore maintained above this level. Error due to high temperatures can also occur in laboratories exposed to the full heat of the sun.

(v) *Self-absorption of radiation.* Self-absorption, that is the absorption of radiation by the sample emitting it, is particularly serious with sources of weak radiation. It can, however, also be important with P^{32} . If the specimen is very thin in relation to the range of the radiation, absorption can be neglected, while if the specimen is very thick, radiation from the lower layers is completely absorbed so that the activity measured is proportional to the concentration of the isotope in the sample. For samples of intermediate thickness more elaborate corrections for self-absorption are necessary. For this purpose a correction curve is prepared.

In liquid-counting corrections are frequently unnecessary. Since the thickness of samples is constant, the counter factor automatically allows for self-absorption if the density of all samples is approximately the same as that of the standard used for calibration. If considerable variations in density occur a correction is, however, made by multiplying the observed counting rates by a factor, e.g.:

Density of sample	1.00	1.05	1.10
Self-absorption correction factor	1.00	1.03	1.07

This factor is empirically determined and will vary between counters depending on the thickness of the counter wall and of the surrounding layer of liquid.

(vi) *Statistical errors.* The time interval between consecutive disintegrations in any sample of a radioactive isotope is subject to random variation and follows the Poisson distribution. Thus the standard error of an estimate of the number of disintegrations occurring in a given period is the square root of this number. The count for a sample is the difference between the observed count and background count. Thus if S counts are recorded from a sample in time, t and B is the background count for the same period, the counting rate of the sample is $(S-B)/t$ and the standard error $(1/t)\sqrt{(S+B)}$. In practice, however, errors will be in excess of this estimate, since the background count cannot be measured at the same time as the sample and it is moreover subject to variations which cannot be estimated statistically.

Errors in assaying are consequently greatest when counting times are short, the counting rate low, and the background high. Examples of the

standard errors for a number of counting rates, times, and backgrounds are:

Observed count (cts./min.)	Background (cts./min.)	Duration of count (min.)	Sample count (cts./min.)	S.E. %
1,000	10	20	900	0.72
	10	10	900	1.06
100	10	20	90	2.61
	10	10	90	3.69
	20	20	80	3.06
50	10	20	40	4.33
	20	20	30	6.24

It is clear that the effect of a high background is particularly great when the counting rate is low. In practice this effect will be greater than the figures indicate, since for purposes of calculation the non-random variations in the background have necessarily been ignored.

In this laboratory limitations in the availability of counting equipment, combined with the necessity of assaying samples within a reasonable period on account of decay, have made it impossible to assay samples for longer than 20 minutes in routine work. Even this length of counting period has placed unfortunate limitations on the replication of experiments. It is therefore apparent that considerable errors have been unavoidable in the assay of samples which give counting rates of 100 per minute or less. Owing to the necessity of using low levels of tracer so that radiation effects are avoided, counting rates in this range are not infrequent, especially when the duration of experiments exceeds 3 or 4 half-lives.

The implications of this situation from the viewpoint of planning tracer investigations will be considered later in this paper.

SAFETY PRECAUTIONS

Safety precautions serve the dual purpose of protecting the health of personnel and preventing contamination which may lead to experimental errors. While the amount of radioactive isotopes likely to be used in a plant physiological laboratory engaged in tracer work is comparatively small, the possibility of injury to the health of workers is always present. Health hazards and the necessary precautions in the handling of isotopes have been discussed in a Medical Research Council manual (1948).

The disposal of radioactive waste may present difficulties. The health of the public must be considered and the maximum quantities it is permissible to dispose of in sewage have been laid down (M.R.C. manual, loc. cit.). In this laboratory waste culture solutions sometimes contain more than the permitted amount (0.1 mC per day or 1.0 mC per week for P^{32}). For disposal such material is precipitated as phosphate (e.g. as barium phosphate) and stored in a container buried in the ground until it has decayed. The precipitate must be handled with care and kept damp so that no particles become airborne. Soil contaminated with P^{32} is stored in metal containers for periods

dictated by its activity and then buried. The disposal of isotopes of long half-life presents a much greater problem—the recommended procedure is to dump them far out at sea.

DISCUSSION

The real value of tracer techniques in research on plant nutrition cannot yet be assessed. It is apparent that new information of many types can be obtained, but the experimental difficulties are still largely undefined and results are few. Certain problems in the organization of research have, however, become apparent in this laboratory, and since they are likely to arise in other investigations, they are here considered. The tentative nature of this discussion must, however, be emphasized.

Unless the half-life of a tracer is very long, the time factor may assume a special importance in limiting the scope of investigations. Analyses must be completed within a period dictated by the activity of the sample and the half-life of the isotope. In nutritional studies with P^{32} these limitations are increased, in some types of work at any rate, by the necessity of using very low activities of tracer to avoid radiation effects. Twelve to sixteen weeks from the commencement of the experiment is frequently the latest time at which the activity of samples is sufficient for convenient routine analysis. Even then, errors due to low counting rates may be considerable. Thus if plants are harvested 10 weeks after treatment with the tracer it may be necessary to carry out all sample preparation and analysis within a fortnight to 6 weeks. When chemical methods are used to assess the results of experiments the analytical work can be carried out over an extended period; as this is impossible in work with short-lived tracers, the degree of replication or the scope of large investigations is likely to be restricted, especially if the experimental periods are long. The effect of this on the value of tracer methods depends on the nature of the investigation. When small or short-term experiments give adequate precision, this restriction may be negligible. For many types of field study it is, however, well recognized that a single long-term experiment of sound design gives greater precision than several small investigations (Fisher, 1947). The substitution of tracer assay for chemical analysis in no way alters statistical requirements, and the new information obtainable by tracer methods must be considered in relation to the resultant loss of experimental precision.

The supply of isotopes may also limit the size of experiments. This is unlikely when P^{32} , I^{131} , or Na^{24} are employed, but high activity preparations of the majority of other isotopes are generally available only in very small amounts.

Against these disadvantages should be balanced the opportunities for new experimental procedures which tracers make possible. The ability to record the changing activity of a single plant while it is still growing opens up wide possibilities. Observations which would otherwise demand the analysis of

a large number of different samples can be made by *in vivo* assay on a single individual. Thus the statistical consequences of random variation can be greatly reduced. Similarly in absorption studies progressive changes in the culture medium can be observed. Equipment for such types of study is, however, at an early stage of development.

It is apparent from the foregoing that in many types of tracer work special importance attaches to the economy of time in laboratory procedures. The sample preparation method here described has been chosen largely with this end in view. One serious limitation should, however, be noted. When the dry weight of samples exceeds 2.0–2.5 g., the volume of extract normally exceeds 10 ml., the capacity of the M.6 counter. Thus only an aliquot can be counted, and counting rates are consequently reduced. If large samples of low activity are to be assayed, the time spent on concentrating the extracts might well be repaid by the consequent reduction in the counting time.

On account of the cost of scaling equipment, economy of time in assay procedures is at least as important as economy of time in sample preparation. Since the standard error of a measurement is inversely related to the counting time, the completion of the assays of an experiment may be possible only at the expense of accuracy. Increases in accuracy without increased counting times can be obtained only by reducing the background count or increasing counter efficiency. This fact emphasizes the importance of avoiding contamination, the chief cause of a high background. Any considerable increase in the efficiency of liquid counters without reduction in the size of sample would, however, seem to be unlikely. Other methods must therefore be considered for increasing the number of assays which can be carried out by a single scaling unit. A larger sample size would have this effect provided the entire extract from a sample could be presented to the counter; automatic sample changing equipment (e.g. Peacock and Good, 1946) can serve the same end by allowing the counter to be used continuously. The former procedure, however, is impracticable in many types of study, while the latter is at the present time likely to be precluded on account of its high cost. In a laboratory working on a comparatively small scale the greatest opportunity for increasing the output of analyses lies in the elimination or rapid rectification of faults.

Though simple to operate, the electronic equipment is complex in design, and when continuously in use the failure of components is not infrequent. The decay of the tracer may increase the consequences of such interruptions. Thus adequate facilities for maintenance are essential. The same considerations emphasize the importance of reliability in the equipment used. The simplification of equipment is an important means to this end, and by reducing costs it may also permit greater replication of equipment. Some directions in which scaling equipment could be simplified, without reducing its suitability for many types of work, have already been discussed, and it is clear that the extent to which tracers can be of value in many types of work is largely dependent on the development of improved equipment and laboratory techniques.

Part I

Tracer methods can yield valid results only if the behaviour of the tracer within the plant is indistinguishable from that of the normal element. The extent to which radioactive isotopes satisfy this requirement must be considered from the following viewpoints:

- (a) their chemical and physical behaviour before disintegration;
- (b) the effects of new substances formed by their disintegration;
- (c) the effects of the radiation they emit.

Whereas, chemically and physically, isotopes with the exception of H^1 and H^3 satisfy this requirement, they may cause significant radiation damage even when supplied in low concentration if they are accumulated metabolically. The investigation of possible radiation effects is therefore an essential preliminary to all physiological investigations.

The value of tracer methods is considered.

Part II

Equipment and laboratory procedures for the electronic assay of P^{32} are described. For the assay of samples containing P^{32} a wet ashing method and the subsequent use of a liquid counting tube is preferred. Improvements of equipment to suit the requirements of plant nutritional studies are considered. Sources of experimental error are examined. On account of the decay of radioactive isotopes, the time factor may be of special importance in tracer work. Its effect on both experimental design and on laboratory procedures is considered. It is stressed that the scope and replication of large experiments, particularly if they are of long duration, may be subject to serious limitations. The importance of simplifying the equipment used in tracer assay and increasing its reliability is emphasized.

ACKNOWLEDGEMENTS

This work has been supported by a grant-in-aid from the Agricultural Research Council, to whom the authors express their gratitude. They are deeply indebted to Professor G. E. Blackman for his constant encouragement and advice.

At all stages in the work they have been greatly dependent on help freely given from many sources. Isotopes have been supplied by the Atomic Energy Research Establishment, Harwell, and, formerly, by the National Institute for Medical Research. Equipment and much valuable advice has been provided by the Director and many members of the staff of the Atomic Energy Research Establishment; Dr. L. H. Gray has given guidance on radiation effects; Dr. Janet Vaughan has undertaken the supervision of health precautions; Mr. N. Veall has given advice on counting equipment. For all this assistance the authors wish to record their sincere thanks.

LITERATURE CITED

- ALLEN, R. J. L. (1940). The estimation of phosphorus. *Biochem. J.* **34**, 858.
- ARNASON, T. J. (1948). Chromosome breakages induced by absorbed radioactive phosphorus. *Cold. Spring Harbour Symposia*, **13**, 1.
- CAMMERY, E., and SPINKS, J. W. T. (1948). Chromosome breakage induced by absorbed radioactive phosphorus, P^{32} . *Canadian J. Res. C*, **26**, 109.
- ARNON, D. I., STOUT, P. R., and SIPOS, F. (1940). Radioactive phosphorus as an indicator of phosphorus absorption of tomato fruits at various stages of development. *Amer. J. Bot.* **27**, 791.
- ARONOFF, S. and CALVIN, M. (1948). Phosphorus turnover and photosynthesis, *Plant Physiol.* **23**, 351.
- ATOMIC ENERGY RESEARCH ESTABLISHMENT (1949). *Radioactive and Stable Isotopes: Catalogue*, Harwell.
- BENSON, A. A., and CALVIN, M. (1948). The path of carbon in photosynthesis. *Cold. Spring Harbour Symposia*, **13**, 6.
- BIDDULPH, O. (1948). Studies of chlorosis using radioiron and radiophosphorus. *Proc. Auburn Conference Radio. Isotopes in Agricultural Research*, p. 90.
- BRITISH RADIOLOGICAL SOCIETY (1947). Certain aspects of the action of radiation on living cells. *Brit. J. Radiol. Suppl.* **1**.
- BROYER, T. C., and HOAGLAND, D. R. (1943). Metabolic activities of roots and their bearing on the relation of upward movement of salts and water in plants. *Amer. J. Bot.* **30**, 261.
- and OVERSTREET, R. (1940). Cation exchange in plant roots in relation to metabolic factors. *Ibid.* **27**, 425.
- CALVIN, M., HEIDELBERGER, C., REID, J. C., TOLBERT, B. M., and YANKWICH, P. E. (1949). *Isotopic Carbon*. New York and London.
- COOKE-YARBOROUGH, E. H., FLORIDA, C. D., and DAVEY, C. N. (1949). An improved quench circuit for Geiger Counters. *J. Sci. Instrum.* **26**, 124.
- DEAN, L. A., NELSON, W. L., MACKENZIE, A. J., ARMIGER, W. H., and HILL, W. L. (1947). Application of tracer technique to studies of phosphatic fertilizer utilization by crops, I. *Soil Sci. Soc. Amer. Proc.* **12**, 107.
- DION, G., BEDFORD, C. F., ST. ARNAUD, R. J., and SPINKS, J. W. T. (1949). Plant injury from phosphorus³². *Nature*, **163**, 906.
- EVANS, R. D. (1947). Radioactivity units and standards. *Nucleonics*, **1** (2), 32.
- FISHER, R. A. (1947). *The Design of Experiments*. Edinburgh, 4th edn.
- GRAY, L. H. (1947). The distribution of the ions resulting from the irradiation of living cells. *Brit. J. Radiol. Suppl.* **1**, 7.
- and READ, J. (1942). The effect of ionizing radiation on the broad bean plant, II. *Brit. J. Radiol.* **15**, 39.
- — (1944). The effect of ionizing radiation on the broad bean plant, VI. *Ibid.* **17**, 271.
- HALL, N. S., and MACKENZIE, A. J. (1947). Measurement of radioactive phosphorus. *Soil Sci. Soc. Amer. Proc.* **12**, 101.
- HENDRICKS, S. B., and DEAN, L. A. (1947). Basic concepts of soil and fertiliser studies with radioactive phosphorus. *Ibid.* 98.
- HEVESY, G. (1948). *Radioactive Indicators*. New York.
- JENNY, H., and OVERSTREET, R. (1938). Contact effects between plant roots and soil colloids. *Proc. Nat. Acad. Sci. Amer.* **24**, 384.
- KAMEN, M. D. (1947). *Radioactive Tracers in Biology*. New York.
- KORFF, S. A. (1946). *Electron and Nuclear Counters*. New York.
- LEA, D. E. (1946). *Actions of Radiations on Living Cells*. Cambridge.
- LEWIS, W. B. (1942). *Electrical Counting*. Cambridge.
- LIBBY, W. F. (1940). Reactions of high energy atoms produced by slow neutron capture. *J. Amer. Chem. Soc.* **62**, 1930.
- (1947). Measurement of radioactive tracers. *Anal. Chem.* **19**, 2.
- MARINELLI, L. D., QUIMBY, E. H., and HINE, G. J. (1948). Dosage determination with radioactive isotopes, I and II. *Nucleonics* **2**(4), 56; **2**(5), 44.
- MEDICAL RESEARCH COUNCIL, 1948. *Introductory Manual on the Control of Health Hazards from Radioactive Materials*. London (reprinted as Appendix B in Atomic Energy Research Establishment, 1949).

- MUIR, R. M. (1942). Effect of radiation from radioactive isotopes on the protoplasm of *Spirogyra*. *J. Cell. Comp. Physiol.* **19**, 244.
- MULLINS, L. J. (1939). The effect of radiation from radioactive indicators on the percolation of ions into *Nitella*. *Ibid.* **14**, 403.
- NELSON, W. L., KRANTZ, B. A., COLWELL, W. E., WOLTZ, W. G., HAWKINS, A., DEAN, L. A., MACKENZIE, A. J., and RUBINS, E. J. (1947). Application of radioactive tracer technique to studies of phosphatic fertilizer utilization by crops, II. *Soil Sci. Soc. Amer. Proc.* **12**, 113.
- OGSTON, A. G. (1948). Interpretation of experiments on metabolic processes using isotope tracer elements. *Nature* **162**, 963.
- OVERSTREET, R., and BROYER, T. C. (1940). The nature of absorption of radioactive isotopes by living tissues as illustrated by experiments with barley plants. *Proc. Nat. Acad. Sci. Amer.* **26**, 16.
- PEACOCK, W. C., and GOOD, W. M. (1946). An automatic sample changer to be used for measuring radioactive samples. *Rev. Sci. Instrum.* **17**, 255.
- PELC, S. R. (1947). Autoradiographic technique. *Nature*, **160**, 749.
- PUTMAN, J. L. (1948). Analysis of spurious counts in Geiger counters. *Proc. Phys. Soc. Lond.* **61**, 312.
- ROTBLAT, J., SAYLE, E. A., and THOMAS, D. G. A. (1948). Scale-of-Hundred counting unit. *J. Sci. Instrum. Phys. Industr.* **25**, 33.
- RUSSELL, R. S., and MARTIN, R. P. (1949). Use of radioactive phosphorus in plant nutritional studies. *Nature*, **163**, 71.
- SANDERS, F. K., and BISHOP, O. N. (1949). Preparation of radioautographs to show the distribution of phosphorus³² in plant tissues. *Ibid.* **163**, 639.
- ADAMS, S. N., and MARTIN, R. P. (1949). Radiation effects due to phosphorus³² in fertilizer experiments. *Ibid.* **164**, 993.
- SPINKS, J. W. T., and BARBER, S. A. (1947). Study of fertilizer uptake using radioactive phosphorus, I. *Sci. Agric.* **27**, 145.
- (1948*a*). Studies of fertilizer uptake using radioactive phosphorus, II. *Ibid.* **28**, 79.
- DION, H. G., READE, M., and DEHM, J. E. (1948*b*). Studies of fertilizer uptake using radioactive phosphorus, III. *Ibid.* **28**, 309.
- CUMMING, E., IRWIN, R. L., and ARNASON, T. J. (1948*c*). Lethal effects of absorbed radioisotopes in plants. *Canadian J. Res.* **C**, **26**, 249.
- THODAY, J. M., and READ, J. (1947). Effect of oxygen on frequency of chromosome aberrations produced by X-rays. *Nature*, **160**, 608.
- THOMAS, W. D. E., and NICHOLAS, D. J. D. (1949). Radioactive phosphorus in biochemical research. *Ibid.* **163**, 719.
- VEALL, N. (1948). A Geiger-Muller counter for measuring the beta-ray activity of liquids, and its application to medical tracer experiments. *Brit. J. Radiol.* **21**, 347.
- WILCOX, P. E. (1949). Isotopic tracer elements in Stereochemistry. *Nature*, **164**, 757.
- YAGODA, H. (1949). *Radioactive Measurements with Nuclear Emulsions*. New York and London.

Factors concerned in the Rooting Responses of Isolated Leaves

F. G. GREGORY AND B. SAMANTARAI

Research Institute of Plant Physiology, Imperial College of Science and Technology, London

INTRODUCTION

INVESTIGATIONS of rooting responses to growth substances have mostly been concerned with root formation in stem tissues in the propagation of cuttings (Pearse, 1948).¹ Although induction of roots in petioles has been described in the literature, no systematic study with isolated leaves has yet appeared. The isolated leaf presents certain advantages, as by its use the mobilization of nutritive factors from the rest of the plant (Stuart, 1938) is avoided, and the activity of the lamina can readily be controlled. The successful establishment of the isolated leaf with an independent root system presents many useful possibilities in physiological researches, some of which have been dealt with by Yarwood (1946): in investigation of assimilation and transpiration it enables experiments to be prolonged without deterioration of the leaf, and in the study of minor element deficiencies changes in the metabolism of the deficient leaf can be followed after supplying the deficient element through the root system. The aims in this work have been (1) to study the factors controlling rooting response of isolated leaves, (2) to study the survival of isolated leaves with adventitious root systems.

I. EFFECT OF DIFFERENT FACTORS ON ROOTING RESPONSE

A. *Previous work*

The older work on rooting of isolated leaves was carried out with the aim of propagation: it has long been known that leaves such as *Bryophyllum* and *Kalanchoë* with preformed buds on the margins of the lamina readily give rise to new plants from these buds. Quantitative studies of such regeneration in *Bryophyllum* were carried out by Loeb (1924). In the case of *Begonia*, on isolated leaves resting on wet sand in a humid atmosphere adventitious buds arise from epidermal cells and give rise to new plants. Lindemuth (1903) cites 28 species whose leaves severed above the axillary bud and inserted in sand form roots from the petiole within 7 to 24 days. He states that leaves of *Citrus* and *Camellia* survived for years without formation of adventitious buds. Winkler (1903) describes root formation from the callus at the cut end and other portions of the petiole of *Torenia asiatica*. Balfour (1912) describes root and bud formation from the callus of the petiole of *Zamioculcas Lodigesii*; root formation takes place freely in *Ficus elastica*, *Camellia*, and *Hoya carnosa*, but bud formation is delayed.

¹ Data presented in this paper were incorporated in a thesis for Ph.D. degree of University of London by B. Samantarai.

Rooting from the petiole in *Viburnum Carlesii* is described by Musgrave (1923) and in *Primula scapigera* by Cooper (1935). Graham (1934) lists plants which can be propagated from leaves; these first form roots and later adventitious buds. In *Brownea*, leaves surviving for 3 to 4 years after rooting are described, and only when senescence of the leaf occurred were adventitious buds produced. Using synthetic growth substances, applied either in lanolin or by irrigating the soil, Zimmerman and Hitchcock (1935, 1937) showed that root formation readily occurred on the petioles of attached leaves of the tomato. They used the rooting response of isolated middle leaves, from tomato plants with 8 or 9 leaves, as a method of estimation of the activity of growth substances. Response occurred after 5–7 days, and also was found in untreated leaves. Graham and Landes (1945) have shown root formation in isolated leaves of *Taraxacum koksaghyz* using α -naphthylacetic acid; they note that very young and old leaves failed to respond. Thimann and Poutasse (1941) obtained root production from petioles of *Phaseolus vulgaris* by treatment with β -indolylacetic acid; no buds were formed. They ascertained the optimal concentration to be 0.025 parts per million, and noted the beneficial effects on rooting of addition of potassium nitrate, adenine, and asparagine. Higher concentrations were found to be toxic, but leaving a portion of stem attached improved rooting and overcame toxicity.

B. Materials and methods

For the present work the leaves of dwarf French bean (*Phaseolus vulgaris*) and ivy (*Hedera helix*) were used. The following growth substances were employed: β -indolylacetic acid (I.A.A.), β -indolylbutyric acid (I.B.A.), and α -naphthylacetic acid (N.A.A.). Preliminary comparisons of these substances at varying concentrations showed that β -indolylacetic acid gave the best results with the bean and α -naphthylacetic acid with the ivy, and these substances were therefore afterwards used.

The method of application finally adopted was as follows. The growth substance was dissolved in a few drops of 95 per cent. alcohol and diluted with distilled water to 400 parts per million (p.p.m.). This was kept as a stock solution in a refrigerator and diluted as required. Though the solution remained fully active for more than 2 months, in practice fresh solutions were made up fortnightly.

The leaves were severed at the base of the petiole round about midday. In the bean leaf the pulvinus was removed as this hindered rooting. The cut ends of the petioles were immersed in the solution to a depth of $1\frac{1}{2}$ in. for 24 hours. On removal from the solutions the ends were washed in tap-water and the leaves were kept in water until rooting had occurred. The bean leaves were kept in humid propagating chambers in full daylight and only lightly shaded from direct sunlight with hessian. The ivy leaves were kept in diffuse light in the laboratory under bell jars. It is important to change the water in which the leaves stand at least once a day.

Except where otherwise stated the bean leaves employed were the second

from the apex, treated for 24 hours in I.B.A. at 2.5 p.p.m. In the ivy either the 9th leaf from the shoot apex or the first leaves to form on axillary shoots were used. The treatment given was 24 hours in N.A.A. at 100 p.p.m.

For each treatment ten replicate leaves constituted the unit, with an equal number of controls. The dimensions of the leaves were recorded at the beginning and end of each experiment. The progress of rooting was noted daily, and the root number recorded. After 15 days in the bean, and 35 days in the ivy, the roots were removed and weighed on a spring balance; the ivy roots were also measured.

C. Experimental results

1. The age of the leaf

Leaves from apex to base of the shoot were simultaneously treated, thus constituting a series of varying age and size. The main axis of the dwarf bean bears four leaves below the terminal inflorescence; from the ivy shoots alternate leaves down to the 13th from the apex were selected. Ten replicates of each were used. The results are shown in Tables I A and B.

TABLE I A

Effect of age on the rooting response of bean leaves after 15 days

	Roots per leaf	1st leaf from apex	2nd leaf from apex	3rd leaf from apex	4th leaf from apex
Treated	Number	102	214	92	32
I.B.A.	Wt. arbitrary				
2.5 p.p.m.	scale units	6.0	20.9	8.3	0.3
Controls	Number	6.8	10.4	4.4	0.2
	Wt. arbitrary				
	scale units	0.3	1.1	0.4	0.03

TABLE I B

Effect of age on the rooting response of ivy leaves after 35 days

Concentration N.A.A. p.p.m.	Roots per 10 leaves	1st leaf from apex	3rd	5th	7th	9th	11th	13th
100	Number	0	40	67	111	141	63	25
	Total length (cm.)	0	10.0	20.8	33.8	41.8	37.3	6.3
50	Number	0	11	38	35	39	31	0
	Total length (cm.)	0	28	13	16.3	21.8	17.8	0

In the bean a well-marked maximum response is seen in both treated and control series in the 2nd leaf from the apex; much less response occurred in the oldest leaf. In the treated 2nd leaf by the 3rd day the upper portion of the petiole was swollen, and in this region particularly roots emerged on the 7th day (Fig. 13). The youngest leaf showed no swelling and rooting was not localized. In the 3rd leaf root emergence began on the 7th day, in the 4th leaf not until the 11th. In the control series roots appeared first on the 2nd leaves after 11 days.

In the ivy a well-marked maximum at the 9th leaf is seen at both concentrations of N.A.A. The untreated leaves showed no rooting at all. Younger leaves than the 3rd in the series showed no rooting as all died after treatment within 7 days. Some of the 3rd leaves also died within 14 days. On the 9th leaf swellings began to appear after 15 days and roots emerged on the 20th day. In the 5th, 7th, and 11th leaves the swellings were delayed until the 18th, 18th, and 25th days respectively.

As a consequence of these experiments the 2nd leaf from the apex in the bean and the 9th leaf in the ivy were used in further work. In the ivy the first leaves to form on the axillary shoots behaved very similarly to the 9th leaves on the main shoots and were therefore largely employed, being more numerous. These leaves on axillary shoots as compared with the 9th leaves on the axis gave the following values per 10 leaves: no. of roots, 9th leaf 145, axillary leaves 198.

2. *Effect of duration of treatment and concentration of growth substance*

The relations between fresh weight or total length of roots and duration of treatment with varying concentrations of growth substance are shown graphically in Figs. 1 and 2 for bean leaves treated with I.B.A. and ivy leaves treated with N.A.A. respectively.

Results with bean leaf. The relation between concentration and duration is nearly linear with 1.25 and 2.5 p.p.m. and the weight of root produced is roughly proportional to the concentration of I.B.A. With short durations, 5 and 10 hours, the greatest effect occurs at highest concentration, but with prolonged exposures rooting becomes less with concentrations above 2.5 p.p.m., indicating a deleterious effect of excess of growth substance. These harmful effects were seen in the reaction of the lamina. At concentrations of 5 p.p.m. the leaves treated for longer than 10 hours tended to roll up, and at the end of the experiment had become green-yellow in colour. With 10 p.p.m. the lamina rolled up even during the period of treatment; within a week the leaves had turned yellow and by the 15th day had shed their leaflets.

Under optimal conditions the petioles showed 6 distinct rows of roots arising between the ridges of the petiole, 4 on the abaxial, 2 on the adaxial flank.

Results with ivy leaf. The range of concentrations of N.A.A. used was much higher than that of I.B.A. used for the bean. The controls failed to root, and below 200 p.p.m. no response followed treatment of 4 hours. Optimal durations occur with 400 and 200 p.p.m., the earlier the higher the concentration. With 100 p.p.m. rooting response increases with duration up to 48 hours, and more or less proportionally. With lower concentrations after 48-hour treatments the response is nearly proportional to the concentration; a similar proportionality holds at higher concentrations up to 12 hours. The optimal concentration of N.A.A. is thus in the region of 100 p.p.m. At the lowest concentrations, 25 and 50 p.p.m., there appears to be a lag in the response up to 16-hour treatment which may be due to the mode of entry of the growth substance into the tissues.

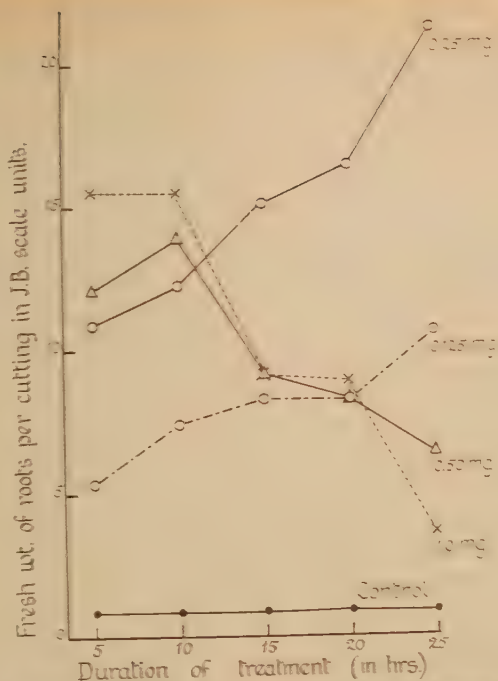


FIG. 1. Rooting response of bean leaves treated with 3-indolyl-butyric acid at various concentrations for different periods. 1.25, 2.5, 5.0, and 10.0 p.p.m. Fresh weights in Joly Balance scale units.

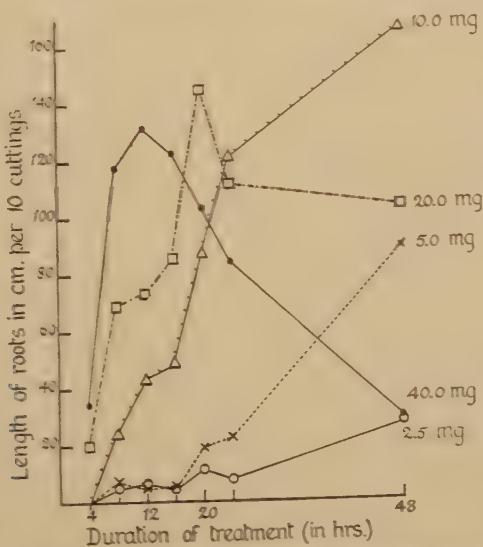


FIG. 2. Rooting response of ivy leaves treated with α-naphthyl-acetic acid at various concentrations for different periods. 25, 50, 100, 200, and 400 p.p.m.

Leaves kept in concentrations of 100 p.p.m. for periods longer than 24 hours showed deleterious effects by yellowing; this adverse effect appeared after 16-hour treatment with 200 p.p.m., and those kept in 400 p.p.m. for longer than 12 hours turned completely yellow in less than a week.

3. Mode of entry of the growth substances

It is known from previous work that growth substances can enter intact stems from lanolin pastes, probably by solution in the cuticle. The mode of treatment used in these experiments made it possible for the growth substance to enter through the vascular system. To ascertain which mode of entry was more effective the following experiments were carried out. Five types of treatment were given. (1) The normal method with the cut petiole immersed in the solution and the leaves exposed to the open air; (2) the leaves were kept during treatment under bell jars with the laminae enclosed between wet filter-papers to check transpiration; (3) the cut ends of the petiole were blocked with vaseline and kept so; (4) the vessels were blocked during treatment but reopened by renewing the cut on transference to water; (5) the petiole was left free in the solution but the main veins of the lamina were cut. Such cutting of the veins has no effect upon transpiration in *Pelargonium* (Mer, 1940), but the phloem is interrupted and therefore translocation of organic materials from the lamina to the petiole is prevented.

Results with bean. The results of the treatments enumerated on the bean and ivy are entered in Table II.

TABLE II

Mode of entry of growth substances into the petiole of isolated leaves

		1. Normal treatment	2. Checked transpiration	3. Blocked vessels	4. Blocked during treatment, later opened	5. Veins cut
<i>Bean</i>	Roots per leaf					
Treated I.B.A.	Number	234	85	51	56	93
2.5 p.p.m.	Wt. arbitrary units	28.5	5.5	5.6	5.5	11.9
Control	Number	9.0	9.1	9.5	9.7	7.2
	Wt. arbitrary units	0.9	1.0	0.98	1.07	0.6
<i>Ivy</i>	Roots per 10 leaves					
Treated N.A.A.	Number	192	33	6	24	48
100 p.p.m.	Wt. arbitrary units	13.5	2.5	0.2	1.3	3.0

In the bean all treatments have led to a reduction in response; the controls show little effect except a reduction due to cutting the veins. Reducing transpiration (treatments 2-4) always leads to a reduction in rooting to about 20 per cent. of the value for normally treated leaves (weight data). Cutting the veins has less effect and about 40 per cent. normal rooting is obtained. The main channel of entry is thus the transpiration stream and the growth substance reaches the tissues through the vascular system; that it has an effect even with blocked vessels proves, however, that some entry can take place through the intact surface of the petiole, though the entry by this means is only one-sixth as fast (weight data). The experiment with veins cut indicates that the phloem plays some part in the reaction for here the transpiration is probably not

reduced, but rooting is reduced by some 60 per cent. which is attributed to the interruption of organic supplies to the petiole from the lamina.

In the ivy checking transpiration has had less effect than blocking the vessels, and the effect of the latter, in this case about 90 per cent. reduction, is even greater than in the bean. The part attributed to the phloem is also greater than in the bean. In the ivy, as usual, no rooting occurred in the controls, again



FIG. 3. Rooting response of bean leaves with the lamina removed from the petiole just before or at various intervals after treatment.

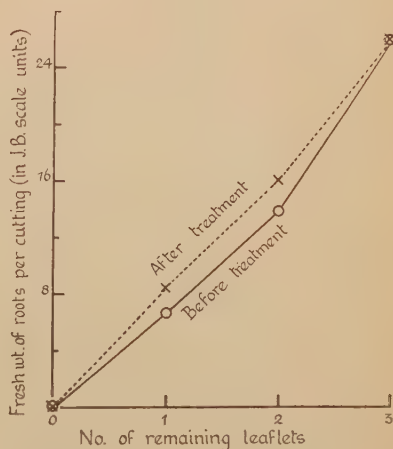


FIG. 4. Rooting response of bean leaves with various numbers of leaflets removed before or after treatment.

showing that with blocked vessels some entry of growth substance can still take place through the cuticle, but only at $\frac{1}{6}$ th the rate of entry through the vessels.

4. Role of the lamina

The part played by the lamina was examined in two ways: (a) the lamina was severed from the petiole just before treatment, or at regular intervals after treatment; (b) the rooting response of the petiole without lamina and with different portions of the lamina left attached was determined.

The results for the bean, in terms of fresh weight of roots, are shown graphically in Figs. 3 and 4. Fig. 3 shows that continued contact of the lamina is a determining factor in the rooting response in the bean. The intensity of response is not proportional to the period of contact, but the effect is greater with each successive time interval up to 15 days. In the controls rooting response fails unless the lamina is kept in contact for 10 days, but then rapidly increases. The actual values of the root weight in controls were, up to 10 days

contact 0, 10 days 0.18 units, 15 days 0.95 units. The rooting response in normal leaves appears after 7 days and the data show that contact of the lamina for less than 7 days has little effect; data to be presented later show that on the 7th day the sugar content of the petiole begins to rise and the total nitrogen content is high up to this time.

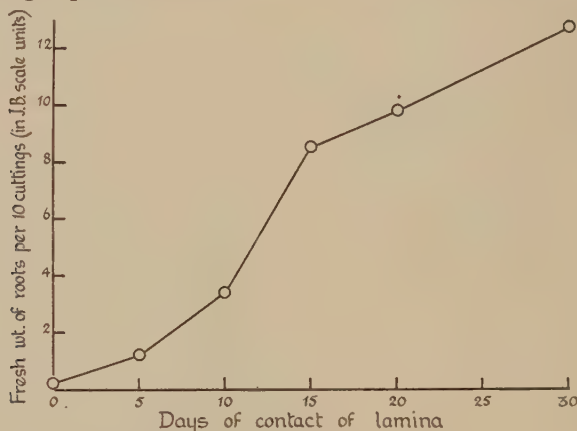


FIG. 5. Rooting response of ivy leaves with the lamina removed from the petiole just before or at various intervals after treatment.

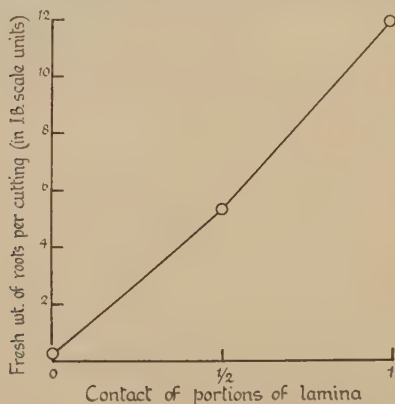


FIG. 6. Rooting response of ivy leaves with varying portions of the lamina removed (before treatment).

Fig. 4 demands little comment. The weight of roots is almost proportional to the leaf area left in contact with the petiole. Removal of the leaflets before treatment has a larger effect and is probably due to the reduction in transpiration during the period of treatment. Without a lamina there is no response, and in the control leaves only when three leaflets remain does any rooting result.

Results for the ivy are shown in Figs. 5 and 6. With increasing duration of

contact of the lamina the response increases progressively up to 15 days, but with longer contacts, up to 30 days, the increment remains constant and is smaller. The data in Fig. 6 show a proportionality between the leaf area remaining on the petiole and the rooting response.

The behaviour of the growth substance in the tissues requires consideration; if growth substance enters the vessels with the transpiration stream it will be carried up into the lamina and unless re-translocation into the petiole can occur the full effect of the total uptake may not appear in the rooting response. The mode of escape from the vessels has not been investigated, but some evidence on active transport of the growth substances in the tissues of leaves can be presented.

5. Movement of growth substance in the leaves

Four series of leaves were examined.

- (a) The petiole was split longitudinally, one half remaining attached to the plant and the other fed with growth substance in the usual way. Control leaves were given water through the detached portion. After treatment the treated half was kept as usual in water.
- (b) The leaf was severed from the plant, the petiole split, and both halves treated with growth substance.
- (c) Leaves similarly treated were given growth substance through one half of the split petiole, the other half in water. Controls for (b) and (c) were kept with the split petiole in water.
- (d) A control series of detached leaves received the usual treatment with growth substance with the petiole entire.

The results are given below in Table III.

TABLE III

Rooting response of leaves with petiole split longitudinally or entire, and either attached to the plant or detached. Weight of roots per leaf

	One half attached to plant, other half treated (a)	Isolated from plant			Both halves treated. Total (b)	Normal treatment (d)
		Half treated with g.s. (c)	Half untreated (c)	Total (c)		
<i>Bean</i>						
Treated with I.B.A. 2.5 p.p.m.	3.13	18.1	0.92	19.0	28.2	26.5
Control	0	—	—	—	1.29	0.99
<i>Ivy</i>						
Treated with N.A.A. 100 p.p.m.	0.18	0.40	0.01	0.41	0.66	1.41

The data for the isolated bean leaf show that the total weight of roots

produced by the split petiole when both halves are treated (*b*) is the same as for the normal leaf with unsplit petiole (*d*); the untreated half (*c*) gives little more root than the untreated controls, and the treated half rather more than half that given by an unsplit petiole. There is thus little evidence for translocation of the growth substance down into the untreated half of the petiole. In the series (*a*) in which half the petiole remained attached to the parent plant and the free end was treated, very little rooting response occurred, far less than in the set of isolated leaves fed through one half only of the petiole. This result is probably due to the translocation of carbohydrates from the lamina away to the rest of the plant rather than into the free end of the petiole. The results for ivy show substantially the same effects; as usual the untreated controls failed to root.

6. Effect of light

Preliminary experiments were carried out with three sets of bean leaves, in full light, in diffuse light in the laboratory, and in complete darkness. In darkness few roots were formed; abscission of the leaflets occurred within 4 days in darkness and 2 days later in dim light. Further experiments were therefore carried out by varying either (*a*) the total quantity of light received or (*b*) the duration of light exposure.

To vary the quantity of light the leaves were placed in cubes of cardboard (18 cm. cube) with apertures of 5, 10, 15 cm. sq. or open at the end. The results for bean are presented in Table IV.

TABLE IV

Rooting response in the bean leaf with varying amounts of light. Roots per leaf

		Aperture size			
Treated I.B.A. 2.5 p.p.m.	Roots per leaf	25 sq. cm.	100 sq. cm.	225 sq. cm.	324 sq. cm.
	Number	10.5	57.9	107	176
	Wt. arbitrary units	0.72	5.6	11.5	17.4
Controls	Number	0.4	1.3	7.0	12.3
	Wt. arbitrary units	0.03	0.05	0.46	0.99

The rooting response is very nearly proportional to the amount of light received in the treated leaves; in the controls the effect on rooting is not linear, but increases with increasing quantity of light.

The results with varying duration of light per day for bean and ivy are entered in Table V.

In the treated bean leaf the weight of roots produced is nearly proportional to the daily illumination between 8 and 12 hours, but from 0 to 4 hours the response is smaller. The number of roots initiated is less influenced by period of illumination: the subsequent growth of root initials is evidently greatly effected by the light dosage. In the control bean leaves, however, both initiation and growth of the roots are much more dependent upon light. In

TABLE V

Rooting response of bean and ivy leaves with varying periods of daily illumination

		Periods of daily illumination			
		0	4 hr.	8 hr.	12 hr.
<i>Bean</i>	Roots per leaf				
	Number	4.3	122	148	194
Treated I.B.A. 2.5 p.p.m.	Wt. arbitrary units	0.03	3.8	12.6	19.3
	Number	0	0.5	9.4	11.8
Control	Wt. arbitrary units	0	0.07	1.03	1.26
	Roots per 10 leaves				
<i>Ivy</i>	Number	4.2	84	173	199
	Wt. arbitrary units	3.5	6.3	11.4	13.4

the ivy both initiation and growth of roots are less dependent upon light than in the bean. The untreated ivy leaves showed no response and are omitted.

7. Effect of temperature

No systematic study of the temperature effect was made. Observations were carried out on bean leaves in October grown in the open air under a bell jar, in a heated frame, and in a heated greenhouse. Similar conditions in January were used for observations on ivy leaves. The results are given in Table VI.

TABLE VI

Effect of temperature on the rooting response of bean and ivy leaves

		Mean midday temperature		
		10.1° C.	13.3° C.	20.3° C.
<i>Bean</i>	No. of roots/leaf	0	7.1	75.0
	Wt. of roots/leaf	0	0.24	8.8
Controls	No. of roots/leaf	0	0	10.4
	Wt. of roots/leaf	0	0	0.89
<i>Ivy</i>	No. of roots/10 leaves	8.0° C.	12.0° C.	17.2° C.
	Length of roots/10 leaves	0	76	214
Treated N.A.A. 100 p.p.m.	No. of roots/10 leaves	0	14.4 cm.	130 cm.
	Length of roots/10 leaves	0		

8. Effect of starvation of the lamina

Experiments with bean leaves. Leaves were removed from the plant and kept for varying periods in darkness before being treated in the usual way. After preliminary starvation the leaves were kept 24 hours in diffuse light in the laboratory while receiving the dose of growth substance and then were returned to full daylight. Control leaves were treated similarly. The leaves starved for 48 hours shed their leaflets during this time; those starved for 36 hours became yellow and shed their leaflets a few days after the treatment. All the other leaves retained their leaflets, but signs of yellowing appeared in those starved for 24 hours.

The results are presented in Table VII and graphically in Fig. 7.

TABLE VII

Rooting response in bean leaves starved for varying periods before receiving growth substance

	Roots per leaf	Periods of starvation in hours				
		0	12	24	36	48
Treated I.B.A. 2.5 p.p.m.	Number	209	157	90.1	55.8	14.5
	Weight	19.1	14.0	9.3	5.8	0.77
Control	Number	13.0	5.2	5.7	0	0
	Weight	1.35	1.09	0.82	0	0

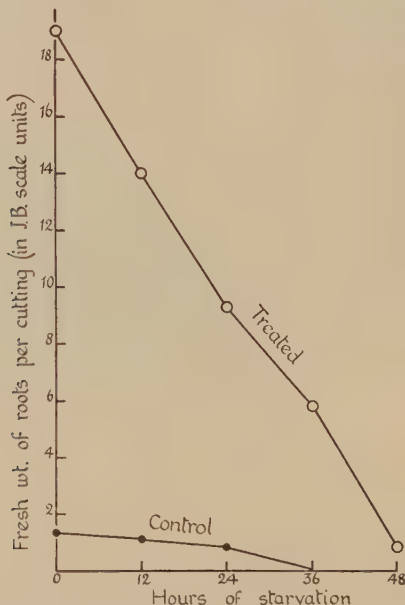


FIG. 7. Rooting response of bean leaves starved for various periods immediately before severance and treatment.

The reduction in rooting up to 48 hours' starvation is almost directly proportional to the duration of darkness both for treated and control leaves; after 48 hours' starvation the rooting response has almost ceased. It will be shown later that the results are closely correlated with the total sugars remaining in the leaves at the time growth substance was given.

Experiments with ivy leaves. The ivy leaf proved much more resistant to starvation than the bean, and the periods of starvation before treatment with growth substance could be extended up to 25 days. Other variations in treatment were introduced into these experiments, viz.

- (a) The leaves were kept in darkness for the specified times and then treated with growth substance (Table VIII, col. a).

- (b) After the period of starvation the leaves were kept in daylight for 4 days before treatment with the growth substance.
- (c) At the time of treatment the leaves were given 1 per cent. sucrose together with the growth substance.
- (d) As (c) with 1 per cent. asparagine.
- (e) As (c) with 1 per cent. sucrose and 1 per cent. asparagine.

In each case half the leaves were kept after treatment in light (A), half in darkness (B).

The results are presented in Table VIII and are shown graphically in Fig. 8.

TABLE VIII

Rooting response of starved leaves of ivy given N.A.A. 100 p.p.m. with or without sucrose and asparagine kept after treatment: A in light, B in darkness.
Results as fresh weight of roots per 10 leaves

Days of starvation			4 days light (b)	1% sucrose (c)	1% asparagine (d)	sucrose + asparagine (e)
0	A	13.4	10.5	13.6	12.2	16.6
	B	3.5	3.8	5.3	5.4	5.6
5	A	5.4	6.4	4.8	6.4	10.0
	B	1.1	2.9	3.2	3.4	3.8
10	A	2.2	1.7	2.7	2.2	3.4
	B	1.1	1.7	2.6	1.8	2.6
15	A	1.9	1.0	2.2	2.2	2.4
	B	0.5	1.0	1.7	1.0	1.2
20	A	0.6	0.3	1.0	0.5	0.6
	B	0.3	1.0	0.4	0.4	0.6
25	A	0	0	0	0	0.3
	B	0	0	0	0	0

The following tendencies are seen in the experimental results:

- (a) With increasing periods of starvation the rooting response decreases in leaves kept both in light and darkness. Exposure to full light after preliminary starvation and treatment with N.A.A. does not increase the rooting response to the level of the unstarved leaves. The effect of light on response becomes progressively less the more the period is prolonged, so that the curves in Fig. 8 approach each other and following 25 days of starvation the response altogether fails in light as in darkness.
- (b) Exposure of the leaves to 4 days of full light after starvation before treatment had no consistent effect on the subsequent response to growth substance in leaves kept in light, but somewhat increases that of leaves afterwards kept in darkness.
- (c) Feeding with 1 per cent. sucrose together with N.A.A. has little effect on leaves kept in light, but on leaves kept in darkness somewhat increases the weight of roots produced. Root number, not entered in the table, fails to show this increase.
- (d) Feeding with 1 per cent. asparagine has no consistent effect in light,

but in the darkened leaves this treatment shows some increase in response.

- (e) Feeding with sucrose and asparagine increases rooting response in both light and dark leaves. Root number shows a similar effect.

The outstanding conclusion to be drawn from this experiment is that the effect of preliminary starvation can only be overcome to a very limited extent

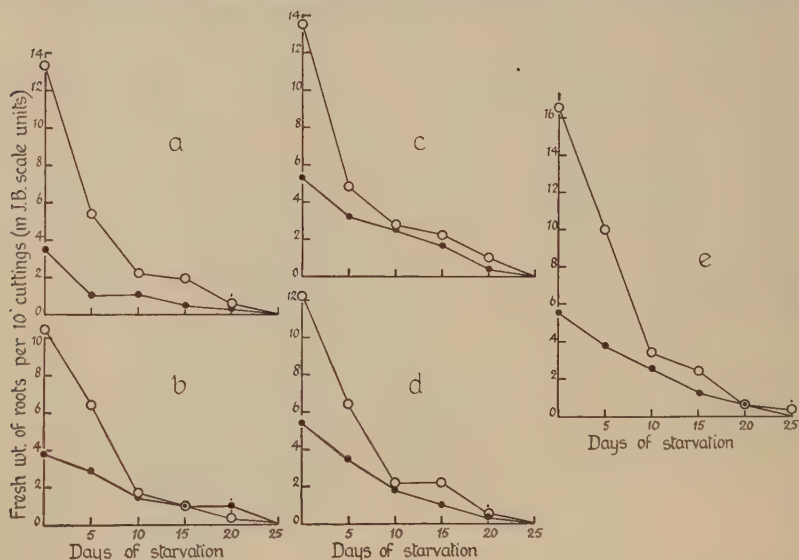


FIG. 8. Rooting response of ivy leaves starved for various periods. Curves with white circles show the response in light and those with black circles in darkness. *a*. Treated with N.A.A. immediately after starvation period. *b*. 4 days in daylight between starvation period and treatment. *c*. Treated with N.A.A. plus 1% sucrose immediately after starvation period. *d*. Treated with N.A.A. plus 1% asparagine immediately after starvation period. *e*. Treated with N.A.A. plus 1% sucrose and 1% asparagine immediately after starvation period.

by subsequent exposure to light and to a slightly greater extent by feeding with sucrose. Feeding with sucrose and asparagine, on the other hand, increases the rooting response of leaves kept in either light or darkness both with and without preliminary starvation, but the additional response obtained by feeding is very small compared with the loss in response due to preliminary starvation.

The sugar content of darkened leaves after varying periods are later shown in Table XVII; by 15 days, the minimum time elapsing before signs of response are detectable in the ivy, the sugar content has fallen by 50 per cent. Preliminary starvation before treatment with N.A.A., thus greatly reduces sugar content. If this is the main effect of starvation, the experiment indicates that the carbohydrate content of the leaf cannot be again increased by subsequent exposure to light either before or after treatment with growth

substance, or by feeding with sucrose. Evidently irreversible changes occur in the leaves kept in darkness, which appear in the yellowing of the lamina; leaves which have developed these symptoms never again become fully green even when fed with sucrose and asparagine.

9. Effect of feeding with sucrose and asparagine

Further work was carried out on the effects of feeding sucrose and asparagine without a preliminary starvation period. In the bean a second feeding was given 5 days after the first, and the experiments were carried out only in light. In the ivy a second feeding was given 7 days after the first, and experiments were carried out in light and in darkness. The results are presented in Tables IX and X.

TABLE IX

Effect of feeding with sucrose and asparagine on the rooting response of bean leaves kept in light. Number and weight of roots per leaf are shown

Feeding material	Treated (I.B.A. 2.5 p.p.m.)		Control	
	Root no.	Root wt.	Root no.	Root wt.
Water	73	7.1	8.7	0.99
1% sucrose	93	11.2	13.7	2.35
2% „	107	11.9	21.1	3.27
0.5% asparagine	94	10.4	30.1	3.93
1% „	87	11.1	21.7	2.94
0.5% asp. + 1% sucrose	112	11.9	39.6	5.54
0.5% „ + 2% „	155	14.8	37.1	4.64
1% „ + 1% „	83	12.9	16.9	2.43

TABLE X

Effect of feeding with sucrose and asparagine on the rooting response of ivy leaves kept in light and darkness. Weight of roots per 10 leaves. No. of roots in brackets

	Treated (N.A.A. 100 p.p.m.) and kept in light				Controls kept in light			
	0	0.5%	1.0%	Mean	0	0.5%	1.0%	Mean
Asparagine								
Sucrose	3.4 (130)	3.6 (118)	9.4 (176)	5.5 (141)	0 (0)	1.0 (12)	0.8 (12)	0.6 (8)
1%	4.6 (134)	3.6 (118)	4.4 (98)	4.2 (117)	0 (2)	0.4 (8)	1.0 (14)	0.5 (8)
2%	6.8 (122)	5.8 (126)	10.4 (154)	7.7 (134)	0 (4)	0.8 (10)	5.6 (52)	2.1 (22)
Mean	4.9 (129)	4.3 (121)	8.1 (143)	—	0 (2)	0.7 (10)	2.5 (26)	—
kept in dark								
Sucrose	0.4 (6)	1.1 (44)	1.1 (46)	0.9 (32)	0 (0)	0.1 (2)	0 (0)	0.03 (0.7)
1%	1.2 (22)	1.3 (52)	1.4 (74)	1.3 (49)	0 (0)	0 (0)	0 (0)	0 (0)
2%	1.2 (40)	1.6 (66)	2.6 (92)	1.8 (66)	0 (0)	0 (0)	0.4 (6)	0.13 (2)
Mean	0.9 (23)	1.3 (54)	1.7 (71)	—	0 (0)	0.03 (0.7)	0.13 (2)	—

In the bean marked response to both sucrose and asparagine was obtained both in treated and control leaves; with the lower concentrations the effects are predominantly additive. 0.5 per cent. asparagine gave the highest response and increase to 1 per cent. reduced the response, both in the absence and presence of 1 per cent. sucrose, except in the weight data for the treated petioles. The combination of 1 per cent. asparagine with 2 per cent. sucrose was not included in the experiment.

In the ivy there were marked effects of feeding both in light and darkness; although as usual no rooting occurred in untreated leaves kept in water, rooting was induced by feeding such leaves, especially with 2 per cent. sucrose plus 1 per cent. asparagine, and even when they were kept in darkness. The average effect of asparagine is somewhat greater than that of sucrose, and in general the increase from 0.5 to 1 per cent. asparagine does not cause a depression of rooting as in the bean data; with 2 per cent. sucrose added the converse effect is striking.

In the bean leaf rooting takes place in leaves not treated with growth substance, and the maximum response of such leaves due to feedings falls below that found in treated leaves kept in water both in respect of number (54 per cent.) and weight of roots (78 per cent.). In the ivy feeding leaves untreated with growth substance in light gives a maximum root weight of 65 per cent. greater than that of treated leaves kept in water, but the number of roots initiated is only 40 per cent. as great. It appears therefore that in the ivy the nutrient factors are more important for root growth than is the case in the bean, although the level of natural growth substance in the bean would appear to be higher, as is indicated by the rooting of leaves untreated with growth substance and confirmed by the much higher concentration of growth substance needed in the ivy to obtain maximum rooting response.

10. Seasonal variation in the rooting response of the ivy

The combined effect of the various external factors upon the rooting of ivy appears in the seasonal variation in rooting. The data are presented in Table XI. Leaves were removed each month throughout the year and treated in the usual way.

TABLE XI

Seasonal variation in the rooting response of isolated leaves of ivy. Number and weight of roots per 10 leaves treated with N.A.A. 100 p.p.m.

Months 1947	Number of roots	Weight of roots
January	0	0
February	0	0
March	49	3.7
April	92	7.6
May	182	14.6
June	187	14.8
July	187	15.8
August	191	14.3
September	164	13.4
October	80	5.7
November	62	4.6
December	0	0

These leaves were kept in an unheated frame outdoors, and under these conditions no rooting occurred between December and February. That the low temperature was the cause of failure was shown by parallel samples kept in a heated greenhouse which showed rooting response at all times.

II. ANALYSIS OF THE LEAF MATERIAL FOR NITROGEN AND TOTAL SUGAR

With a view to further investigation of the nutritional factors in the rooting response, systematic analyses were carried out of the total and soluble nitrogen fractions and total sugars in laminae and petioles of the leaves before treatment and at regular intervals after treatment.

A. Materials and methods

The leaves were divided into lamina and petiole on the various sampling occasions: for nitrogen estimations the material was dried at 80° C. for 48 hours and was afterwards ground and passed through a 60-mesh sieve. For sugar analysis the leaves were plunged into boiling 95 per cent. ethyl alcohol, and refluxed twice for 3 hours, each time with fresh quantities of alcohol. The combined alcoholic extracts together with 30 ml. distilled water were distilled at reduced pressure at 30–40° C. to remove all alcohol. The remaining extract in water was then made up to 100 ml. or 50 ml. according to the amounts of material used. These extracts were centrifuged to remove insoluble material and the clear solution remaining used for sugar estimation.

Total nitrogen was estimated by the microkjeldahl method. For estimating soluble nitrogen the proteins were precipitated by grinding twice with a 3 per cent. solution of trichloroacetic acid and centrifuging to obtain a clear solution. The clear extract was then analysed for nitrogen.

Total sugar was estimated by the method of Harding and Downs as modified by van der Plank (1936). The non-fermentable sugar reducing fraction was determined after fermentation with 10 per cent. suspension of baker's yeast. Sucrose was hydrolysed by invertase in the original extract. The difference in reducing powers after invertase action and after yeast fermentation gave the reducing power of the glucose equivalent of the total sugar.

B. Results

1. Variations in nitrogen and sugar during the rooting response

(a) *Nitrogen changes in the bean leaf.* The data for the bean leaf are entered in Table XII and shown graphically in Figs. 9 and 10; data for petiole and lamina are separately presented.

The variation in the dry weight of the leaves sampled on different occasions makes a direct comparison of the total amounts of the fractions impossible; conclusions can only be drawn from the percentage composition of the dry matter. The water-content changes are complex. In the control laminae the water content steadily increases; in the treated the water content reaches a maximum 3 days after treatment. In the petioles the reverse is seen: the control petioles show a continuous fall in water content, which occurs to a less extent in the treated. Such a change in the petioles may be expected if rapid translocation of materials from the lamina takes place.

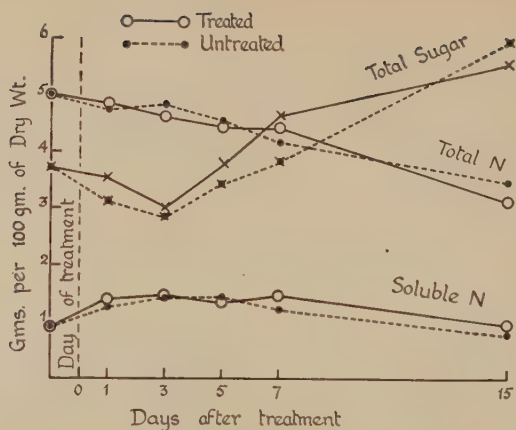


FIG. 9. Percentage content of total nitrogen, soluble nitrogen, and total sugar in the lamina of the bean leaf before, and at regular intervals after, severance and treatment.

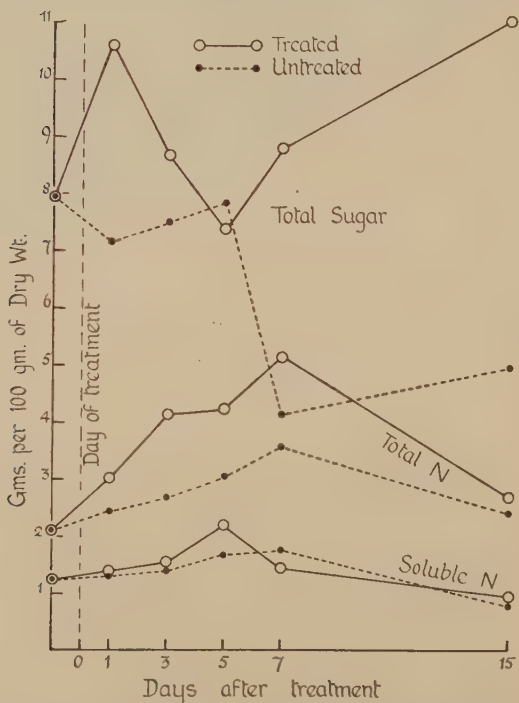


FIG. 10. Percentage content of total nitrogen, soluble nitrogen, and total sugar in the petiole of the bean leaf before, and at regular intervals after, severance and treatment.

TABLE XII

Variation in dry weight, nitrogen, and sugar in the lamina of the bean leaf during rooting response. (I.B.A. 2.5 p.p.m.)

	Dry wt. as % fresh wt.	Total N % dry wt.	Soluble N % dry wt.	Ratio sol./insol. N	Total sugar % dry wt.
<i>Treated leaves</i>					
Before treatment	13.88	4.98	0.91	0.22	3.72
1 day after treatment	12.33	4.85	1.39	0.40	3.56
3 days " "	10.95	4.62	1.45	0.45	3.01
5 " " "	11.67	4.46	1.33	0.42	3.83
7 " " "	13.16	4.43	1.46	0.49	4.64
15 " " "	13.08	3.19	0.94	0.41	5.60
45 " " "	15.0	4.50	1.07	0.31	3.60
<i>Untreated leaves</i>					
1 day after isolation	13.29	4.79	1.25	0.35	3.13
3 days " "	12.50	4.82	1.41	0.41	2.86
5 " " "	12.56	4.57	1.42	0.45	3.45
7 " " "	12.23	4.19	1.22	0.41	3.84
15 " " "	12.14	3.49	0.77	0.28	5.99

TABLE XIII

Variations in dry weight, nitrogen, and sugar in the petiole of the bean leaf during rooting response. (I.B.A. 2.5 p.p.m.)

	Dry wt. as % fresh wt.	Total N % dry wt.	Soluble N % dry wt.	Ratio sol./insol. N	Total sugar % dry wt.
<i>Treated leaves</i>					
Before treatment	9.73	2.11	1.26	1.48	7.94
1 day after treatment	9.94	3.02	1.39	0.85	10.62
3 days " "	9.16	4.14	1.55	0.60	8.68
5 " " "	9.63	4.26	2.20	1.06	7.36
7 " " "	9.80	5.15	1.46	0.40	8.80
15 " " "	10.31	2.70	0.94	0.54	11.03
<i>Untreated leaves</i>					
1 day after isolation	10.0	2.43	1.32	1.19	7.17
3 days " "	11.4	2.69	1.41	1.10	7.49
5 " " "	12.3	3.09	1.69	1.20	7.85
7 " " "	14.6	3.59	1.76	0.96	4.12
15 " " "	16.3	2.40	0.79	0.49	4.97

The percentage total nitrogen in the dry matter of the laminae both in control and treated leaves falls steadily. The percentage of soluble nitrogen in the lamina increases markedly in both series in the 1st day after treatment, which accompanied by a fall in total nitrogen indicates hydrolysis of protein. The ratio of the soluble to insoluble nitrogen confirms this. Within 1 day after isolation of the leaves both treated and untreated show a large increase in the ratio, and this is maintained to the 7th day, at which time the root initials are emerging in the petioles.

From the 1st to the 7th day little further change in soluble nitrogen occurs in either series. By this time the roots have emerged and the large loss in both soluble and total nitrogen from this time onwards is related to the rapid

growth of the roots. Since the leaves were kept in water, nitrogen for synthesis in root growth must have been derived from the cutting. After the 15th day the rooted leaves were transferred to soil and 30 days later analysis showed that the nitrogen fractions had returned nearly to their original values by absorption and metabolism of nitrogen from the soil. It is interesting to record that nodules were formed on the roots of the isolated leaves when planted out in soil.

In the petiole of the bean the picture presented is very different (Fig. 10 and Table XIII). The total nitrogen increases immediately after isolation in the control leaves and much more so in those treated with I.B.A. The rise in total N continues in both series up to the 7th day, after which a rapid decline sets in, and by the 15th day the values in both series approach the original value before isolation. The changes in soluble N resemble closely those in total N, but the relative changes are different, as is seen from the ratios of soluble/insoluble N. Soluble nitrogen decreases relative to insoluble nitrogen, particularly so in the treated leaves. From the data it may be concluded that isolating the leaf leads to rapid hydrolysis of protein in the lamina and translocation to the petiole. In the petiole re-synthesis of protein takes place so rapidly that the soluble N is less in relation to protein N than in the leaf attached to the plant. The changes in relative soluble N are related to the initiation of root primordia which occurs between the 1st and 7th days. In the treated petioles the fluctuations in the ratio of soluble N to protein N run parallel with changes in starch content as assessed by iodine coloration.

(b) *Nitrogen changes in the ivy leaf.* The changes in soluble and insoluble nitrogen in treated and untreated leaves of the ivy are presented graphically in Figs. 11 and 12. In Fig. 11 the changes in the lamina are shown. Total nitrogen as percentage of dry matter in the control leaves changes little until the 15th day after treatment and then slowly falls. In the treated leaves total nitrogen falls steadily to the 25th day. Soluble nitrogen in both sets tends to increase, and the ratio of soluble to insoluble N rises considerably: from 0.16 to 0.24 on the 20th day in the controls, and 0.16 to 0.33 on the 15th day in the treated leaves, followed in both series by a fall on the 25th day to 0.20 in the control and 0.26 in the treated laminae.

In the petioles of the control leaves both nitrogen fractions remain remarkably constant and the ratio of soluble to insoluble N shows no trend. In the treated petioles, on the other hand, the percentage of total nitrogen steadily rises up to 20 days, while the soluble nitrogen has doubled by the 15th day and then falls rapidly. The ratio of soluble to insoluble N shows a marked increase from 0.37 to 0.81 on the 15th day followed by a rapid decline to 0.28 on the 25th day.

The control leaves failed to root in these experiments and thus no meristematic activity is found in such petioles; the steady values of the percentage nitrogen fractions indicate that no marked changes took place under the conditions of the experiment. In the treated petioles the same trends are seen as in the bean petiole, though in the ivy the changes are much slower in agreement

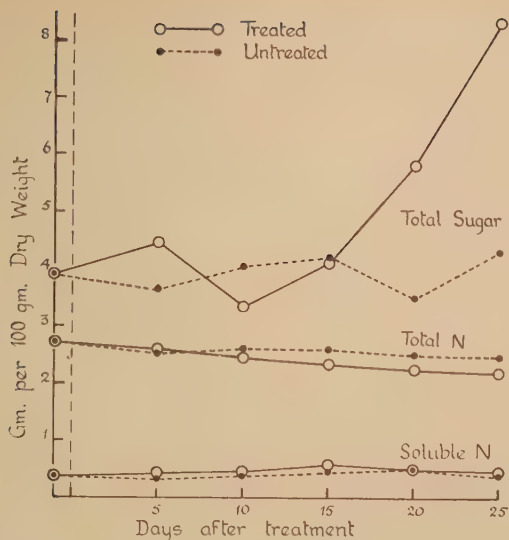


FIG. 11. Percentage content of total nitrogen, soluble nitrogen, and total sugar in the lamina of the ivy leaf before, and at regular intervals after, severance and treatment.

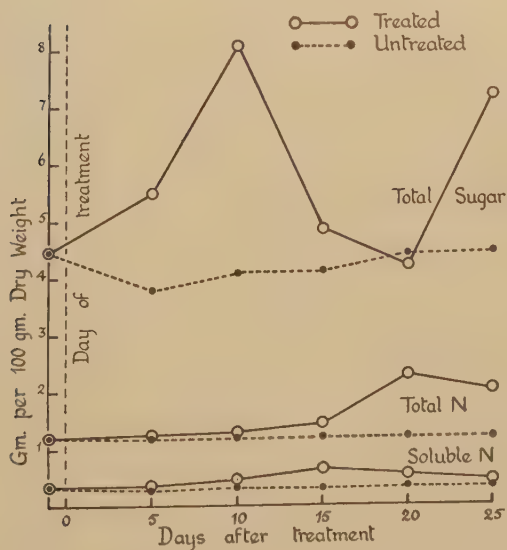


FIG. 12. Percentage content of total nitrogen, soluble nitrogen, and total sugar in the petiole of the ivy leaf before, and at regular intervals after, severance and treatment.

with the times taken for the roots to emerge in the two plants, viz. 6 to 7 days in the bean and 20 to 23 days in the ivy. The changes in total nitrogen as percentage of dry matter for the leaf as a whole are shown in Table XIV.

TABLE XIV

Percentage total nitrogen in the whole leaf (lamina and petiole together) of the bean and ivy before and after treatment with growth substance

Bean				Ivy			
		Control	Treated			Control	Treated
Before isolation		4.66		Before isolation		2.47	
1 day after isolation		4.53	4.55	5 days after isolation		2.30	2.37
3 days	" "	4.50	4.55	10 "	" "	2.35	2.23
5 "	" "	4.33	4.50	15 "	" "	2.30	2.16
7 "	" "	4.06	4.59	20 "	" "	2.22	2.26
15 "	" "	3.06	2.84	25 "	" "	2.20	2.17

The data indicate that both in bean and ivy until the emergence of adventitious roots (bean 7 days, ivy 20 days) the nitrogen content remains very nearly constant. There is therefore no evidence for a loss of nitrogen during the hydrolysis and re-synthesis of proteins such as Pearsall and Billimoria (1939) have described in leaves.

(c) *Changes in sugar content in the bean leaf.* The data for total sugar (excluding starch) in the bean leaf during the rooting response have been presented in Tables XII and XIII and graphically in Figs. 9 and 10. Both in treated and untreated leaves a similar course of change is shown by the lamina. One day after isolation and treatment the sugar content has fallen and reaches a minimum on the 3rd day, after which the sugar content rises to the end of the experiment. The sugar content in the treated leaves is, except for the last value, consistently higher than in the controls. The initial fall in sugar content in the treated leaves accompanies a marked fall in the starch content as estimated by iodine staining, but the conspicuous rise in sugar after the 3rd day is accompanied by only a small increase in starch, and on the 15th day, when the sugar content is much higher than in the leaf before treatment, the starch content remains much lower. In the untreated leaves iodine staining revealed no marked changes in starch content, which remained high throughout.

The percentage sugar content in the petiole is consistently higher than in the lamina up to the 7th day in control and treated leaves. The petioles of the treated leaves are markedly richer in sugar than the controls. One day after treatment the petiole shows a great increase, but sugar content then declines to reach a value on the 5th day almost identical with the value before treatment. In the control leaves up to the 5th day sugar content remains fairly constant. These variations in sugar content are clearly related to the changes in starch already referred to. A rapid hydrolysis of the starch occurs in the lamina of the treated leaves and the sugars thus formed, as well as preformed sugar, are translocated to the petiole; no such hydrolysis occurs in the controls. After the 5th day the behaviour of the treated and control leaves diverges strikingly:

in the control petioles the level falls to a low value and remains low, whereas in the treated the sugar content continues to rise.

Later work in progress has shown by direct analysis that starch in the treated leaves (lamina and petiole) is much lower than in the controls, whereas the reverse relation holds for total sugar.

(d) *Changes in sugar content in the ivy leaf.* The changes in total sugar content in the ivy leaf are graphically presented in Figs. 11 and 12. In the treated leaves the changes in the lamina and petiole somewhat resemble those already discussed in the bean leaf, though spread over a longer period; the control leaves, however, show little change in sugar in either lamina or petiole. Up to 15 days no consistent change in the laminae of treated leaves is seen, but thereafter a rapid increase in sugar takes place. Tests with iodine showed an abundance of starch before treatment and up to the 5th day when a decrease began; after the 10th day there was much less starch. In the control laminae starch content remained almost at the original level till the 10th day and only later showed a fall.

The preliminary rise and fall in sugar content of the petioles of treated leaves of bean (Fig. 10) and ivy (Fig. 12) required 5 days in the former and 20 days in the latter, in each case corresponding with the period of initiation of roots, and just less than the time taken for root emergence. The difference in behaviour of the control leaves of bean and ivy only occurs after root initiation is complete, and the rapid decline in sugar content seen in the bean is absent in the ivy. This difference in reaction is due to the failure of root initiation in the ivy, while in the bean roots are belatedly formed by the control leaves.

2. *Effect of age of the leaf upon nitrogen and sugar content during the rooting response*

The rooting responses of bean leaves of varying age have already been presented in Table I A. The analyses for such leaves at different times after treatment are given in Table XV.

The variations of the fractions in all the leaves, so far as the incomplete data permit such comparison, conform to the same pattern. In the lamina nitrogen content falls steadily, in the petiole it first rises and then falls; an accumulation of total sugars occurs. Well-marked gradients are seen from apical to basal leaves: in the lamina (and in the leaf as a whole) the maximal nitrogen content occurs in the youngest leaf and the maximal sugar content in the oldest. The superior rooting of the 2nd leaf may perhaps be connected with its high initial total sugar combined with the high nitrogen content, although neither separately is quite maximal. Reference may here be made to the great increase in rooting caused by feeding with 2 per cent. sucrose plus asparagine (Table IX). In the ivy the gradients are less marked. The 9th leaf which roots most profusely shows maximal sugar (except for the 1st leaf which fails to root; see Table I B) and its nitrogen content is only exceeded appreciably in the 13th leaf where the sugar content is very low. The ivy data are not presented.

TABLE XV

Percentage of total nitrogen and total sugars in the dry matter of bean leaves of varying age at different stages in rooting. (I.B.A. 2.5 p.p.m.)

	1st leaf from apex		2nd leaf from apex		3rd leaf from apex		4th leaf from apex	
	Petiole	Lamina	Petiole	Lamina	Petiole	Lamina	Petiole	Lamina
% total N in dry matter								
Before treatment	3.11	6.22	2.65	5.67	2.26	5.06	2.14	3.66
1 day after treatment	—	—	3.98	5.31	—	—	—	—
3 days " "	3.97	5.39	4.45	4.98	4.47	4.55	4.05	1.94
5 " " "	—	—	4.80	4.40	—	—	—	—
7 " " "	3.15	4.60	4.34	3.70	4.20	4.10	—	—
% total sugar in dry matter								
Before treatment	8.05	2.52	7.95	3.98	3.81	2.97	3.72	6.01
1 day after treatment	—	—	9.30	3.61	—	—	—	—
3 days " "	8.07	3.77	6.90	3.79	7.18	5.74	7.90	7.49
5 " " "	—	—	8.02	5.16	—	—	—	—
7 " " "	11.94	4.90	8.08	8.44	—	—	—	—

3. Effects of varying the daily periods of illumination on nitrogen and sugar contents of bean leaves during the rooting response

The results of the analyses are given in Table XVI; the rooting responses have already been presented in Table V. The changes in nitrogen content somewhat resemble those seen in Table XV (the same data being embodied for the 2nd leaf and 12 hours light). The rooting response does not show any obvious relation with the amount of nitrogen translocated to the petiole in the different treatments, but the total sugar content at 7 days in the petiole and lamina is evidently related to the rooting response.

TABLE XVI

Percentage of total nitrogen and total sugars in the dry matter of bean leaves exposed to varying periods of daily light at different stages of rooting. (I.B.A. 2.5 p.p.m.)

	No light		4 hrs. light		8 hrs. light		12 hrs. light	
	Petiole	Lamina	Petiole	Lamina	Petiole	Lamina	Petiole	Lamina
% total N in dry matter								
Before treatment	2.65	5.67	2.65	5.67	2.65	5.67	2.65	5.67
3 days after treatment	3.49	4.79	3.48	4.00	3.35	3.84	4.45	4.98
7 " " "	3.83	—	3.53	3.96	3.21	3.72	4.34	3.70
% total sugar in dry matter								
Before treatment	7.95	3.98	7.95	3.98	7.95	3.98	7.95	3.98
3 days after treatment	3.13	1.40	7.00	8.40	7.50	9.50	6.90	7.79
7 " " "	1.80	—	4.50	4.60	6.80	7.00	8.08	8.44

4. Effect of starvation on the total nitrogen and sugar contents of the leaf

The rooting responses of these leaves have already been presented in Table VII for the bean and Table VIII for the ivy. Data for the leaves starved for various periods just prior to treatment with the growth substances are given in Table XVII.

TABLE XVII

Percentage of total nitrogen and sugar in the dry matter of starved leaves at the time of treatment with growth substance

	% N in dry matter		% total sugar in dry matter	
	Petiole	Lamina	Petiole	Lamina
<i>Bean</i>				
No starvation	2.65	5.67	7.95	3.98
12 hrs. starvation	2.50	5.53	6.22	3.12
24 "	2.41	5.42	4.78	1.78
48 "	2.41	5.30	2.32	0.51
<i>Ivy</i>				
No starvation	1.59	2.89	7.20	6.70
5 days starvation	1.53	2.79	6.22	4.22
10 "	1.51	2.57	4.63	3.44
15 "	1.15	2.54	3.89	3.13
20 "	1.10	2.64	2.85	2.36
25 "	1.12	2.33	1.86	2.66

A similar picture is presented by both types of leaves; the rooting response is closely related to the sugar content of the leaves at the time of treatment with growth substance at the end of the starvation period. There are no data for the sugar contents of the leaves after replacing in full light, so that it is not possible to state whether the failure in the rooting response after starvation is due to failure in sugar synthesis.

III. GROWTH OF THE LAMINA OF ISOLATED LEAVES AFTER ROOTING AND PLANTING IN SOIL

After treatment some of the leaves were planted in soil. Under these conditions the bean leaves survived the leaves on the parent plants, while the ivy leaves have survived in a healthy condition for more than 2 years. Yarwood (1946) in his review mentions the fact that rooted ivy leaves have survived a period of 6 years. How the rooting was achieved is not stated: they were certainly not stimulated by any growth substance. There is mention in the literature of leaves isolated and rooted growing to a size larger than those left on the plant.

The series of leaves of different ages taken in succession from the ivy shoot showed, however, that the serial order of size remained after planting out. This may be seen from Fig. 14, which is a photograph taken 1 year after planting out, and it is evident that the original variation in size among the leaves has been maintained. A study of the growth of the rooted leaves was made in the course of this work.

Data for the bean are entered in Table XVIII, which shows the dimensions of 6 groups of leaves, 4 isolated and 2 attached to the plant.

No marked difference in growth was noted as between the treated and untreated isolated leaves; both, however, showed much less growth than similar untreated leaves attached to the plant.



FIG. 13. Roots developed in treated and untreated bean leaves, taken from the 2nd node from the apex, 12 days after removal.



FIG. 14. Ivy leaves, taken from successive nodes from apex towards base of the shoot as indicated by the numbers, rooted and kept in soil for 1 year.

TABLE XVIII

Growth of isolated rooted leaves of the bean compared with similar attached leaves. Means of ten leaves

Treatment	Average initial length of a leaflet (cm.)	Average initial breadth of a leaflet (cm.)	Average increment (45 days)	
			length (cm.)	breadth (cm.)
Isolated and treated . . .	5.81	2.43	1.15	0.74
Isolated not treated . . .	5.66	2.52	1.19	0.76
Isolated with portion of stem and treated . . .	5.82	2.60	1.37	0.85
Isolated with portion of stem and untreated . . .	6.01	2.57	1.38	0.75
Attached to plant and treated	5.81	2.60	3.93	2.19
Attached to plant untreated	5.73	2.47	6.93	3.33

To test whether any influence was exerted by stem tissues, leaves were also employed in which small pieces of stem were removed with the petioles. These again showed little difference from the isolated leaves.

Some of the leaves attached to the plant were induced to root on the petiole by applying growth substance in alcohol solution to the petioles which were afterwards wrapped in wet cotton-wool. This series showed markedly less growth than untreated attached leaves but much more than isolated leaves.

The following conclusions may be drawn.

Isolating the leaf greatly reduces the capacity for further growth. As these leaves had ample root systems and were planted in soil in which nodules were produced it seems unlikely that the leaves were short either of water or nutrients. The small portion of stem left attached to the leaf increased to a minor extent the capacity for further growth, but in no way comparable to that of attached leaves. Application of growth substance to the petiole of attached leaves and the formation of adventitious roots markedly reduced the capacity of the leaves to grow.

It might thus be supposed that the application of I.B.A. exerted some inhibition on leaf growth, but this cannot be the case as the behaviour of isolated untreated leaves shows. Further it may be supposed that at the time of isolation cell division was complete but cell extension was still going on. If this was the case the latter process must have been inhibited. As cell extension is regarded as a major function of natural auxins, and as much work has shown that these substances diffuse from cut petioles, it appears unlikely that lack of natural auxins was a cause of the arrest in leaf expansion. The whole matter is obscure and further work requires to be done to elucidate the position.

The effect of isolating the leaf is much more striking in the ivy (Table XIX).

TABLE XIX

Growth of isolated rooted ivy leaves of different ages

Leaf position on shoot	5 Sept. 1946		15 June 1948	
	Length (cm.)	Breadth (cm.)	Length (cm.)	Breadth (cm.)
3rd	4.0	6.0	4.2	6.2
5th	4.1	5.6	4.1	5.8
7th	4.6	6.1	4.7	6.1
9th	6.1	8.3	6.4	8.5
11th	7.5	10.5	7.7	10.8
13th	10.2	16.3	10.2	16.4

The data show that in leaves of all sizes and from all positions on the shoot no growth has taken place in nearly 2 years after isolation. Meanwhile, however, the adventitious root system has reached considerable dimensions as seen in Fig. 15. At this stage of the work nothing definite can be said to account for these challenging facts.



FIG. 15. Ivy leaves taken from 9th node from apex, rooted, and kept in soil for 1 year.

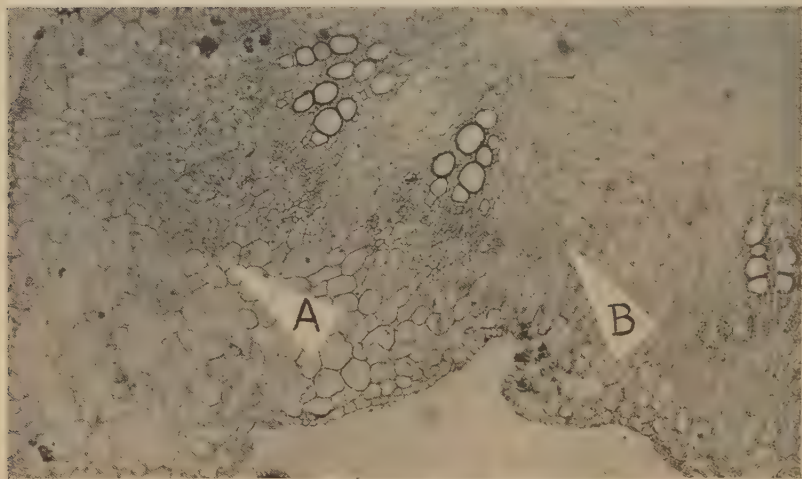


FIG. 16. Transverse section of bean petiole 3 days after treatment. A. Cells outside the phloem becoming meristematic. B. Cells in the medullary rays becoming meristematic.

IV. HISTOLOGICAL CHANGES IN ISOLATED LEAVES OF BEAN AND IVY ACCOMPANYING THE FORMATION OF ROOTS

Petioles were fixed before treatment and at regular intervals afterwards in a modified Navashin fluid and embedded in paraffin. After sectioning they were stained with safranin and haematoxylin.

Development of roots in the bean petiole

In the bean petiole the bundles are arranged in a ring, larger bundles below the ridges and smaller in the furrows. Three days after treatment parenchymatous cells just outside the phloem and medullary ray cells enlarge and show divisions mainly periclinal forming meristematic regions in the inner cortex (Fig. 16). By the 4th day the phloem is proliferating and smaller adventitious bundles are forming in the medullary rays. The organization of root primordia

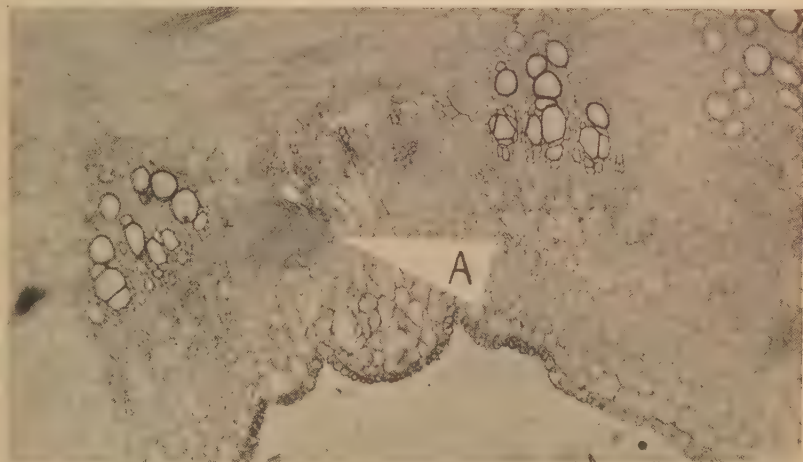


FIG. 17. Bean petiole, 5 days after treatment. A. Root primordium formed in the medullary ray derivatives.

by groups of cells of the medullary rays takes place very rapidly (Fig. 17). On the 6th day roots are seen growing through the disorganized cortical tissues (Fig. 18), and fully emerged roots appear on the 7th day. The process of root formation is thus completed in the bean in 5 to 6 days.

Development of roots in the ivy petiole

The bundles in the ivy petiole constitute a ring, and a layer of cambium is present between xylem and phloem (Fig. 19). The activity after treatment with growth substances is localized in the ivy and not widespread as in the bean. The parenchymatous cells of the bundle sheath just outside the phloem become meristematic and undergo periclinal divisions towards the 15th day after treatment, and the cambium layer is seen to be in division (Fig. 20). The derivatives of the bundle sheath rapidly divide and multiply and form root primordia by the 20th day (Fig. 21). The central zones of these primordia rapidly differentiate into vascular tissue which establishes connexion with the primary vascular bundles either by activity of the cambium or directly through the primary phloem.

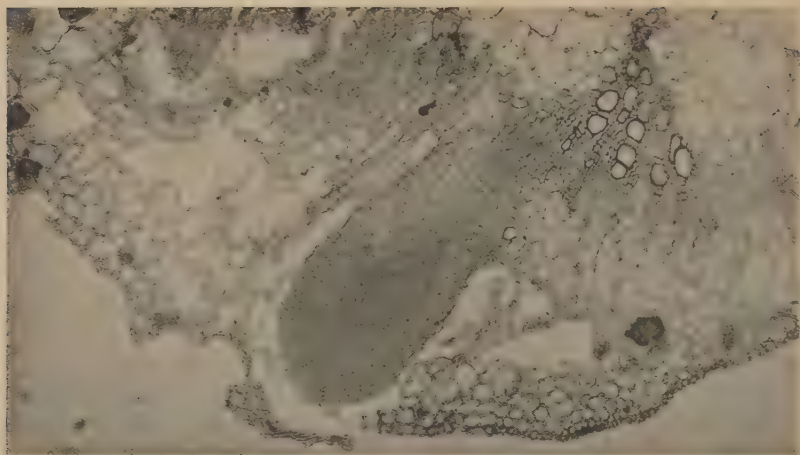


FIG. 18. Bean petiole, 6 days after treatment. The adventitious roots are joining up with the adjacent vascular bundles.

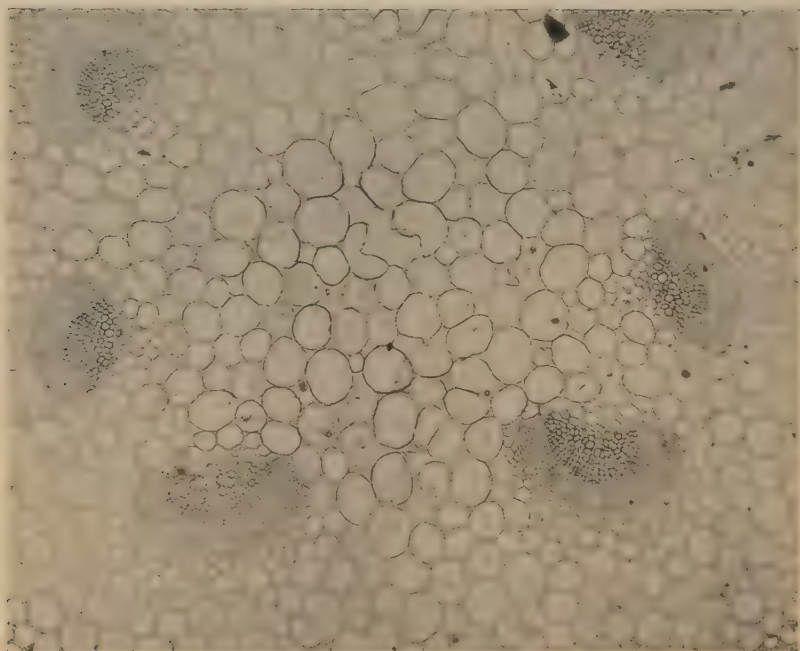


FIG. 19. Transverse section of ivy petiole before treatment.

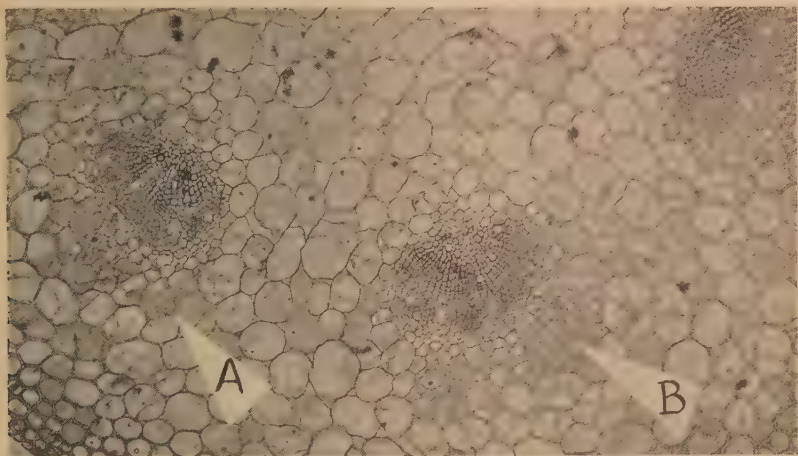


FIG. 20. Ivy petiole, 15 days after treatment. A. Cells of the bundle sheath becoming meristematic. B. Derivatives formed from bundle sheath.

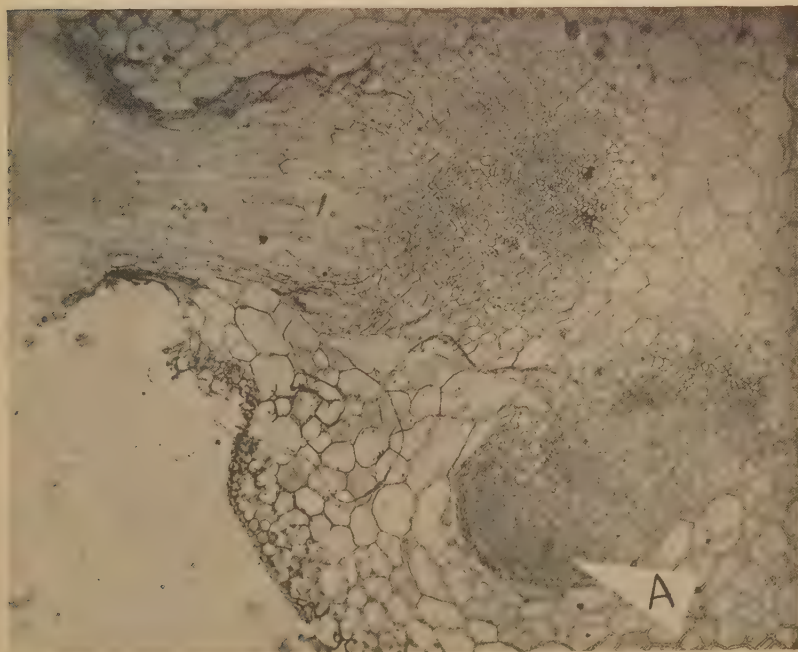


FIG. 21. Ivy petiole, 20 days after treatment. A. Root primordium.

GENERAL DISCUSSION

The rooting response in the cuttings of isolated leaves comprises three stages:

1. Enlargement of certain cells in the petiole, de-differentiation and cell division.
2. Organization of groups of cells thus formed into root initials.
3. Growth of the primordia into mature roots.

The first stage in the process obviously requires the presence of a cell-extension hormone, and this in the extending region behind the meristem is associated with the action of auxin. It is also a characteristic action of many of the synthetic hormones such as those used in this work, but whether the effect of these substances is direct or, on the other hand, whether the presence of the synthetic hormone merely stimulates the production of natural hormone in the tissue, is still uncertain.

The localization of the reaction, as seen in the transverse sections of the petioles, presents certain peculiarities, however, for cell division begins outside the vascular bundles but in proximity to them. Evidence has been presented (Table II) that the growth substance primarily enters the tissues from the xylem, and yet it is not within the vascular bundle that cell enlargement and cell division begin. The picture presented demonstrates that some diffusion process is concerned, the hormone diffusing into the tissues from the vascular strand. The fact that cell division is associated particularly with the cells adjacent to the phloem merely demonstrates that sugars and soluble nitrogen are required and these presumably diffuse from the sieve tubes. There is some evidence that the reactivity of the cells of the parenchyma and starch sheath vary, some being readily stimulated; thus in the bean the ray cells are particularly active. Whether this is due to differences in permeability to the growth substance is unknown and requires experimental investigation.

The evidence presented in this paper leaves no doubt as to the primary importance of sugar in the initiation of the primordia. The experiments recorded on removal of the lamina, starvation, and variation in light in both amount and duration, all bear witness to this. The feeding experiments also indicate the importance of nitrogen. In the bean sufficient natural growth substance is synthesized in the lamina to permit of some root initiation even without the application of any synthetic growth substance, but the reaction is intensified by feeding with sucrose and asparagine. In the ivy no response occurs naturally on isolation of the leaf, but again by feeding with sucrose and asparagine, root formation results even without the application of growth substance, especially when both nutrients are supplied at high level. In the ivy, then, nutrient supply is a limiting factor in the response to a greater extent than in the bean. The marked reduction in rooting seen in the control bean leaves when asparagine is increased from 0.5 to 1 per cent., with sucrose supplied in low concentration or absent, does not occur in the ivy and may be related to the normally much higher nitrogen content of the bean leaf.

The factors concerned in the organization of the root primordium are entirely unknown. The process occurs with great rapidity and is associated with an active synthesis of protein. Data have been presented to show that feeding with the growth substance leads to rapid starch hydrolysis in the lamina and to a less extent proteolysis. Soluble nitrogen is translocated to the petiole together with the hydrolytic products of starch and the ratios of soluble to insoluble nitrogen show that rapid protein synthesis occurs in the petiole. The discrepancies between the root number and root weight indicate that, while soluble nitrogen is available, root initiation proceeds rapidly, but in some of the primordia extension into roots is limited. The transverse sections of the petiole show root primordia in all stages of growth from uncoordinated groups of meristematic cells to fully emerged roots. Active competition for nutrients must therefore take place amongst the primordia formed. The extent to which nitrogen and sugar limit the number of roots formed cannot be stated from the experiments presented here, for the sugar and nitrogen were given twice only. To elucidate this further will require continual feeding experiments which have not been done. In general, the results obtained in this work confirm those of other investigators who employed shoots for their experiments.

The emergence of roots through the cortical tissues requires further examination. The method whereby the tissues are penetrated by the roots is not at all clear. There is no sign in the sections of cells crushed and therefore active digestion by excretion from the root or possibly by autolysis must occur.

Some of the other experiments performed require further comment. It is generally believed that the natural hormones are translocated either in the phloem or through the parenchyma tissues of plants. There is ample evidence to show that transport is usually polar and occurs only towards the base of the plant. The evidence in Table II is compatible with some transport in the phloem, but the experiments reported in Table III in which the petiole was split and one half was treated indicate that the growth substance which is carried up into the lamina in the transpiration stream is not again exported to the other half of the petiole. It is known from the coleoptile test that β -indolyl-butyric acid moves in the coleoptile tissues, but no evidence of such movement from the leaf has been found in this work. The effects of the growth substance in the lamina are seen by the rapid starch hydrolysis. It is also noted that starch is never again synthesized to any great extent in the lamina of the treated leaves, and analysis for starch has since confirmed this effect in the bean leaf. The after effect of a period of darkness before the application of the growth substance on root initiation (Tables VII and VIII, Figs. 7, 8) also presents a problem which requires further work.

The effect of leaf age on the rooting response shows that in general the intensity of the reaction is controlled by the metabolic rates in the leaves particularly with regard to sugar supply. The failure to respond of very young ivy leaves is not apparently due, however, to low photosynthetic activity, for the 1st leaf shows maximal sugar (p. 181), nor would this account for the lethal

effect on such leaves. It is probable that the optimum concentration will be different with different ages, though this has not been tested. It is, however, interesting that β -indolyl-butyric acid acts on the very young leaves like the toxic phenoxy compounds used in weed control.

The work here reported is of a preliminary nature and the outstanding problem is the failure of the rooted leaf to grow to the normal size. This may open up interesting lines of work on the nature of the leaf growth hormone. At present nothing can be said on the matter and speculation would be premature. The use of the technique for rooting leaves presents many interesting possibilities for physiological research and its application in many fields will be most valuable.

SUMMARY

The rooting responses of isolated leaves of the dwarf French bean and the ivy were studied by applying solutions of growth substances to the petiole: β -indolyl-butyric acid (I.B.A.) for the former and α -naphthyl-acetic acid (N.A.A.) for the latter.

The following factors were investigated:

1. The variation in response of successive leaves from the apex towards the base of the plant.
2. The optimal concentrations of the growth substances and the relation between duration of application and concentration.
3. The relation of age of the leaf and optimal concentration (ivy).
4. The nutritive factors concerned in the response were studied, (a) by varying periods of contact between lamina and petiole, (b) by varying duration of light, (c) by varying the amount of light, and (d) by feeding with sucrose and asparagine.

The most responsive leaf in the bean was the 2nd from the apex, in the ivy the 9th leaf. Very young leaves were killed. With petioles immersed for 24 hours the optimal concentrations were I.B.A. at 2.5 parts per million (p.p.m.) for the bean, and N.A.A. 100 p.p.m. for the ivy. It was shown that the active substance entered mainly through the cut end in the transpiration stream; entry through the cuticle was much slower.

Starvation of the cuttings whether by low light intensity, darkness, or separation of the lamina from the petiole reduced root formation. Feeding with sucrose and asparagine increased the response.

Analysis of the leaves was carried out at regular intervals for total sugar, total and soluble nitrogen. In the bean soluble nitrogen increased slightly in the petiole during the first 5 days after treatment, after which insoluble nitrogen continued to accumulate until the 7th day, when the roots first emerged. After this the total nitrogen decreased. Sugar content of the petiole at first increased but rapidly fell before the roots emerged and then again increased. In the ivy petiole the increase in soluble and total nitrogen was very slow, the former being greatest on the 15th day and the latter on the 20th day after treatment. The total sugar increased to the 10th day, but then decreased

until the emergence of roots, after which it again increased. Treated leaves of the bean and ivy of different ages and the leaves starved for varying periods were also analysed for total sugars and total nitrogen. Treated bean leaves receiving varying periods of daily illumination were also analysed to elucidate their rooting response.

Histological examination showed that in the bean the roots arose in the rays between the vascular bundles and in the ivy external to the vascular bundles opposite the phloem.

The growth of the rooted leaves was followed for more than a month in the bean and over one year in the ivy. Some growth was found in the immature bean leaves which survived treatment, but the final size was much below that of the control leaves attached to the plant. In the ivy very little further growth occurred although the leaves produced a large root system and after 2 years are still alive: the original differences in size of the successive leaves at the time of removal from the plant still remain.

LITERATURE CITED

- BALFOUR, J. B. (1912). Problems of propagation. *J. Roy. Hort. Soc.* **37**, 457.
 COOPER, R. E. (1935). Alpine garden. *Gardener's Chron.* March, p. 155.
 GRAHAM, R. J. D. (1934). The work of Laurence Baxter Stewart. *Trans. and Proc. Bot. Soc. Edinburgh*, **31**, 450.
 GRAHAM, P. R., and LANDES, M. L. (1945). Investigations on rubber bearing plants. I. Propagation of *Taraxacum koksaghyz* by means of leaf cuttings. *Bot. Gaz.* **108**, 260.
 LINDEMUTH, M. H. (1903). Notes and abstracts. *J. Roy. Hort. Soc.* **28**, 697.
 LOEB, J. (1924). *Regeneration*. McGraw Hill, New York.
 MER, C. L. (1940). The factors determining the resistance to the movement of water in the leaf. *Ann. Bot. N.S.* **4**, 397.
 MUSGRAVE, C. T. (1923). Methods of propagation in an amateur garden. *J. Roy. Hort. Soc.* **48**, 194.
 PEARSALL, W. H., and BILLIMORIA, M. C. (1939). The influence of light upon nitrogen metabolism in detached leaves. *Ann. Bot. N.S.* **3**, 601.
 PEARSE, H. L. (1948). *Growth substances and their practical importance in horticulture*. Tech. Comm. No. 20, Imp. Bur. Hort. and Plantation Crops.
 STUART, N. W. (1938). Nitrogen and carbohydrate metabolism of kidney bean cuttings as affected by treatment with indole acetic acid. *Bot. Gaz.* **100**, 298.
 THIMANN, K. V., and POUTASSE, E. F. (1941). Factors affecting root formation of *Phaseolus vulgaris*. *Plant Physiol.* **16**, 585.
 VAN DER PLANK, J. E. (1936). The estimation of sugars in the leaf of the mangold (*Beta vulgaris*). *Biochem. J.* **30**, 457.
 WINKLER, H. (1903). Über regenerative Sprossbildung auf den Blättern von *Torenia asiatica* L. *Ber. d. bot. Ges.* **21**, 96.
 YARWOOD, C. E. (1946). Detached leaf culture. *Bot. Rev.* **12**, 1.
 ZIMMERMANN, P. W., and HITCHCOCK, A. E. (1935). Absorption and movement of synthetic growth substances from soil as indicated by the response of aerial parts. *Contrib. Boyce Thompson Inst.* **7**, 447.
 ——— (1937). Comparative effectiveness of acids, esters, and salts as growth substances and methods of evaluating them. *Ibid.* **8**, 337.

Studies in the Metabolism of Mould Fungi

I. A PRELIMINARY STUDY OF THE METABOLISM OF CARBON, NITROGEN, AND SULPHUR BY *ASPERGILLUS NIDULANS*

D. J. D. HOCKENHULL¹

Faculty of Technology, University of Manchester

THE following study on the metabolism of *Aspergillus nidulans* was undertaken as background to a more detailed study on the metabolism of sulphur in relation to cystine-synthesis. It was of the same type as that carried out with *Penicillium notatum* (Hockenhull, 1946).

EXPERIMENTAL

Composition of medium. The chemically defined medium was made up from: (all quantities as % w/v) glucose, B.P. 5, NaNO_3 0.5, Na_2SO_4 0.1, KCl 0.1, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.1, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.05, FeCl_3 0.005, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.002, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.002, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.002, ZnCl_2 0.002, NaF (pure) 0.001 in tap-water. The constituents were analytical reagent quality unless otherwise stated. The medium was adjusted to a pH value between 5.8 and 6.0 with NaOH before sterilization.

Strain of organism. *Aspergillus nidulans* A 69, kindly given to the author by Mr. Edward Yuill, was used throughout.

Growth conditions. Twenty-five ml. of medium was placed in each 100-ml. cotton-wool-plugged conical Pyrex flasks. Steam-sterilization was carried out at 20 lb. per sq. in. for 10 minutes. The *inoculum* was prepared from 7-day-old cultures of the mould on $2\frac{1}{2}$ per cent. agar slopes of the above medium (for deficient strains 0.025 per cent. cystine was added) in 6 in. $\times$ 1 in. tubes. These were shaken with 10 ml. of a 0.1 per cent. dilution of Calsolene Oil H.S. (I.C.I. Ltd.) in water. (The inoculum for the sulphur-metabolism experiments was made up in distilled water only.) The resultant suspension after counting an aliquot in a haemocytometer cell was diluted with sterile water to give about 10^7 spores per ml. The inoculum was 0.5 ml. per flask.

After inoculation the flasks were randomized and incubated at $30^\circ \pm 0.5^\circ$. Five flasks were removed for each day's analyses.

Sampling. In Expt. 1 the liquor was filtered from the felt of 5 flasks. The felts were washed collectively with about 100 ml. water and the washings added to the first filtrate. Five ml. conc. HCl was added to the combined filtrates to prevent further fermentative changes, and the whole diluted to 250 ml. ($2 \times$).

In Expt. 2 a smaller quantity of water was used for washing the felts. The combined filtrates were made up to 150 ml. ($1.2 \times$). The non-acidified filtrate was stored at 5°C , a little ether being added as preservative.

¹ Present address: Messrs. Glaxo Laboratories Ltd., Fermentations Division, Barnard Castle, Co. Durham.

Methods of analysis. The analyses of total-liquor-carbon, total-felt-carbon, reducing-sugar, volatile-base-nitrogen, nitrate, and phosphate were carried out as Hockenhull (1946). Total-nitrogen was carried out by microkjeldahl determination on 2 ml. of filtrate after treatment with Zn dust and dilute H_2SO_4 to reduce nitrate to ammonium salts. Volatile acid (as acetic) was determined by titration with baryta against phenol red after distillation in steam from acidified solution. Oxalate was determined as the Ca salt by an unpublished, improved micromethod due to my colleague, Mr. G. Halliwell.

Analysis for free sulphate was carried out by treating 10 ml. of the culture filtrate diluted to contain about 1 mg. sulphur with 5 drops 0.2N. HCl, 2 ml. 2 per cent. aqueous benzidine hydrochloride, and 4 ml. acetone, in this order, and standing for 2 hours at 5° (adapted from a method of Rosenheim and Drummond described by Hawk (1938)). The precipitated benzidine sulphate was filtered on a Gooch crucible, washed with saturated benzidine sulphate solution, redissolved in a little hot water, and titrated with 0.01N. NaOH against phenolphthalein. Free-plus-ethereal-sulphate was determined by the same precipitation after 5 ml. filtrate had been evaporated to dryness with 0.5 ml. conc. HCl at 100° and redissolved in 5×2 ml. water. In the analysis for total sulphur 5 ml. culture filtrate (about 1 mg. S) was evaporated without ebullition together with 2 ml. conc. HNO_3 and 0.5 ml. Benedict's reagent (10 per cent. cupric nitrate, 2.5 per cent. KClO_3 (w/v in water)) in a silica crucible. When almost dry a further 1 ml. conc. HNO_3 was added and evaporation continued to dryness, when the temperature was gradually raised until the crucible was at dull redness. After cooling, 1 ml. conc. HCl was added to dissolve the CuO and to decompose remaining nitrate. The mixture was evaporated to dryness and redissolved in 5×2 ml. water. Sulphate was precipitated as above. Powdered mycelial matter was more difficult to analyse. 200–250 mg. were heated with 4 ml. conc. HNO_3 and 0.5 ml. Benedict's reagent. After the volume had fallen to 0.5–1 ml. a further 4 ml. conc. HNO_3 was added and the combustion then completed as for culture filtrate. No difficulties due to precipitation of phosphate were encountered with these techniques, which were rather simpler than those described in an earlier paper (Hockenhull, 1948).

Seitz-filtrates of culture filtrates were further analysed to determine the amount of substances capable of stimulating the growth of deficient strains of *Asp. nidulans* (sulphiteless, γ , and thiosulphateless, ν) derived from A 69 by mustard-gas mutation (Hockenhull, 1948, 1949). The growth-responses of these strains to culture filtrate were compared with those to cystine by a technique to be described later.

RESULTS

Expt. 1. Carbon and Nitrogen

As Fig. 1 indicates, the carbon utilization was slow until day 5, when the liquor-carbon fell rapidly until day 8 to a minimum value of about 15 per cent. of the original. The utilization of glucose paralleled this. Its concentration fell swiftly to about 10 per cent. of original by day 10, and gradually to zero by day 20. The mycelium-carbon rose quickly after day 5 to a maximum (about 57 per cent. of initial carbon) on days 8 and 9, and then fell off gradually throughout.

Negligible quantities of small-molecule products accumulated. The highest

amounts of oxalic acid were 0.020 (day 20) and 0.010 mg. per ml. (day 12). There was no systematic rise and fall of the quantities of this acid in the culture filtrate. No other dicarboxylic or hydroxy acids were detected. A small quantity of volatile acid was found (see Table).

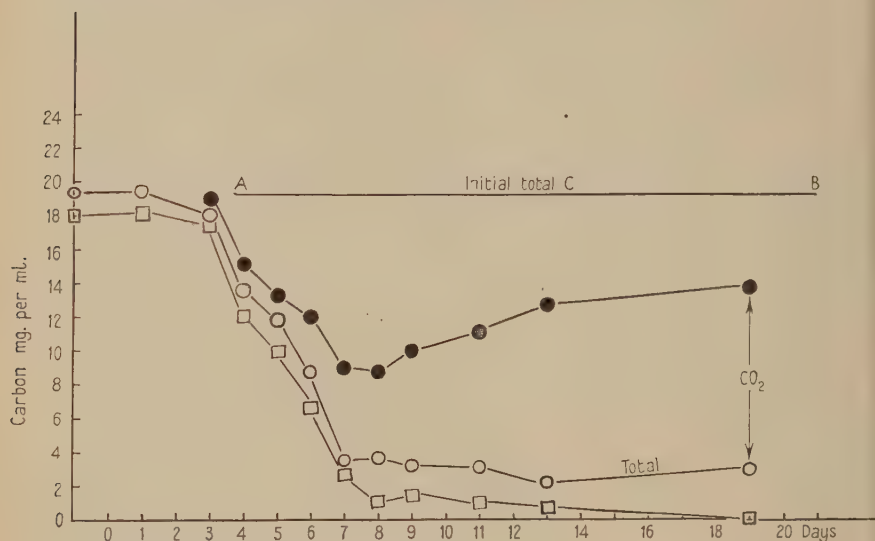


FIG. 1. Carbon-metabolism of *Asp. nidulans*: Expt. 1. 25 ml./100 ml. conical flask. ○ = total carbon in medium; □ = carbon as reducing sugar (glucose); ● = carbon in mycelium measured down from AB which represents total initial carbon.

The production of volatile acid by *Asp. nidulans*

Day	4	5	6	7	8	9	10	12	14
Volatile acid (as acetate), mg. C per l.	6	21	45	60	94	76	50	41	20
Felt weight, mg. C per ml.	0.5	4.2	5.9	7.4	10.5	10.6	9.5	8.2	6.6

This, however, represented a negligible proportion of the carbon supplied in the medium (about 20 mg. per ml.).

A very small quantity of some substance giving a positive iodoform reaction was present in a neutral steam-distillate of the medium.

As Fig. 2 indicates, the nitrogen metabolism at first paralleled that of the carbon. However, although nitrate had completely disappeared by day 8, the total nitrogen in the culture filtrate reached a minimum value of about 10 per cent. of the original by day 10 and then rose slowly to a steady value of 20 per cent. of the original by day 14. This slight release of nitrogen by the felt was insignificant in comparison to the fall in felt-carbon between days 9 and 20, so that, as Fig. 2 shows, the nitrogen content of the felt rose steadily.

No volatile base was detected in the liquor up to day 14, a very small quantity appearing on day 20.

The pH value of the liquor rose sharply after day 3 (pH 6) to pH 8.1 on day 7 and then more slowly to reach pH 9.0 on day 20.

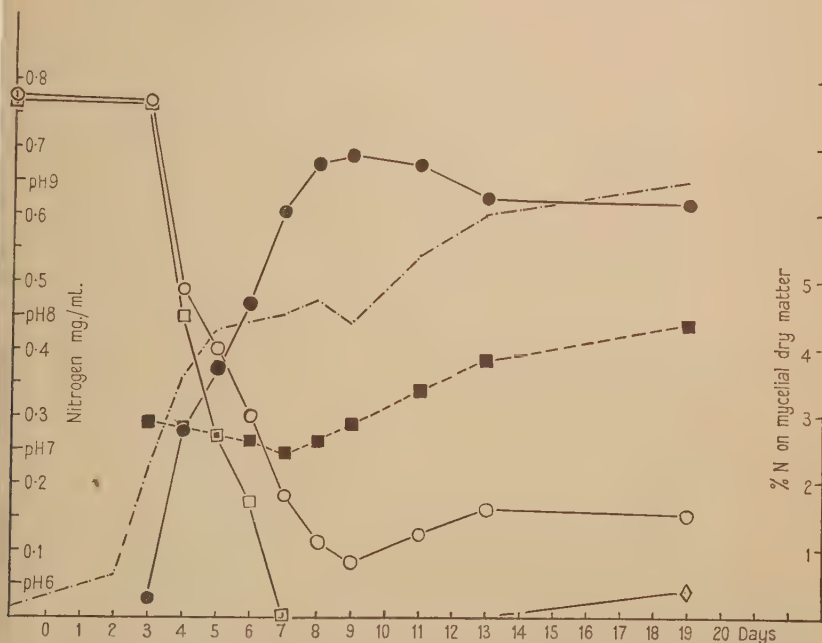


FIG. 2. Nitrogen-metabolism and pH changes with *Asp. nidulans*: Expt. 1. 25 ml./100 ml. conical flask. ○ = total nitrogen in medium; □ = nitrate nitrogen; ● = mycelial nitrogen; ◇ = volatile-base nitrogen; ■ = % nitrogen on mycelial dry matter; - - - = pH.

Rough experiments indicated a fall in liquor phosphate to a minimum at day 10 followed by a rise to 60 per cent. of the original value by day 14.

Expt. 2. Sulphur

Fig. 3 shows that the general metabolic picture is the same as that described in Expt. 1. The total sulphur in the culture filtrate began to fall rapidly at day 4, reaching a minimum of about 60 per cent. of original by day 10, which remained constant to day 20. The level of free sulphate sulphur was the same as the total sulphur and the sulphate after hydrolysis within the limits of analytical error. In spite of the rapid fall in mycelial dry matter after day 8 there was excretion of sulphur from the felt into the liquor. The maintenance of the total amount of felt-sulphur (0.11 mg. per ml.) during the period of decline thus paralleled that of nitrogen in the first experiment (0.6–0.7 mg. per ml.).

The absence of a non-sulphate-sulphur fraction in the filtrate shown by the chemical analyses was further confirmed by the bio-assay which has been

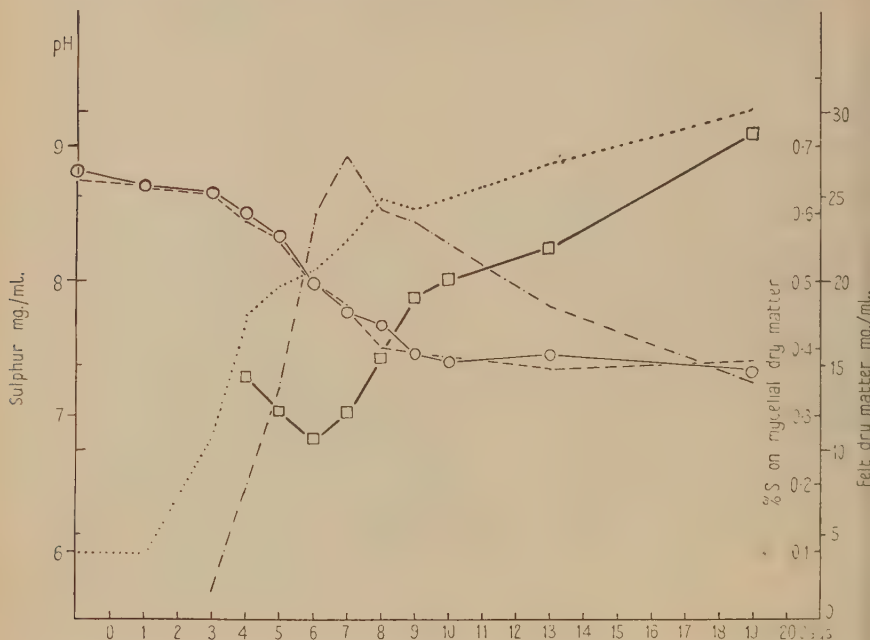


FIG. 3. Sulphur-metabolism of *Asp. nidulans*: Expt. 2. 25 ml./100 ml. conical flask. ○ = total sulphur in medium; --- = sulphate sulphur in medium; □ = % sulphur on mycelial dry matter; = pH; -.-.- = mycelial dry weight.

shown capable of detecting with certitude 6 mg. per l. of available sulphur (initial 260 mg. per l. as sulphate). Qualitative tests for cystine and thiosulphate were also negative.

DISCUSSION

The main products of the metabolism of *Asp. nidulans* A 69 on the medium employed were mycelium and CO_2 . From rough calculation (taking mycelium protein as $6.3 \times$ nitrogen, and the remainder as cellulose) it appears that the synthesis of felt material accounts for about 50% of the thermal energy yielded by the combustion of glucose to CO_2 . Comparing the above results with those obtained by Raistrick (1931) with other strains of *Asp. nidulans*, it is seen that here oxidation was much more complete, differences being due perhaps to strain of organism, to unrestricted aeration in the former experiments, to higher temperature (30° as against Raistrick's 22 – 25°), higher nitrate (0.5 per cent. as against 0.2 per cent. NaNO_3) and to the wider range of trace elements used. The percentage carbon in the felts was also much lower (40–43 per cent.

as against 46–50 per cent.), which differences might, however, be due to the extended duration of Raistrick's experiments.

The occurrence of a small quantity of volatile acid, presumably acetic acid, in the culture filtrate may, in view of the possibility of its importance in the intermediate metabolism of the mould, be compared with the results of Chughtai, Pearce, and Walker (1947), who isolated acetic acid from the felts of four strains of *Asp. niger*, the yields per volume of medium being of the same order.

Striking features of the nitrogen-metabolism were the absence of volatile-base formation (especially free ammonia directly from nitrate) and the great stability of the nitrogenous fraction of the mycelium. This latter effect was paralleled by the behaviour of the felt-sulphur. This might indicate that the two elements were metabolically connected, e.g. that the sulphur were bound as a protein constituent. The ratio (0.11 mg. S to 0.6–0.7 mg. N) points to a ratio of 1 sulphur atom to about 15 nitrogen atoms in the felt.

The absence of non-sulphate-sulphur fractions in the culture filtrate is interesting as Raistrick and Vincent (1948) have found an accumulation of acid-hydrolysable sulphate with other *Aspergilli* grown on a rather similar medium. Their growth periods were, however, rather more protracted than those of the author.

The small amount of sulphur taken in by the felt is comparable to that taken in by *P. notatum* on similar media, especially if allowance be made for the lower felt yields with the latter organism (Hockenhull, 1948). The mould shows similarities also to *P. notatum* (unpublished work) with respect to the lack of small-molecule fermentation products, the attainment of high pH values, and the low carbon content of the dried felt (40–43 per cent.). *Asp. nidulans* produced, however, a much greater yield of mycelial matter (25 as against 9 mg. per ml. maximum), although the nitrogen content was less (2.5 as against 5.6 per cent. on day of maximum felt-weight).

The rise in pH noted in the present experiments might be attributed to (1) reduction of nitrate with release of kation, (2) assimilation of phosphate with release of kation, (3) non-accumulation of acid fermentation-products, but not to any important degree by formation of free ammonia.

SUMMARY

The growth and metabolism of a normal strain (A 69) of *Aspergillus nidulans* upon a simple chemically defined medium has been followed analytically. The main metabolic products were mycelial material, which reached its maximum after about 8 days, and CO₂. Negligible quantities of small-molecule carbon-compounds were detected. A minute amount of volatile acid which was maximal at the time of maximal felt-weight was found. Nitrate, the sole nitrogen-source, was all utilized. The felt took up most of the nitrogen and retained it in spite of the decrease in mycelial matter due to senescence. Negligible amounts of volatile-base nitrogen were formed. Sulphate was partly assimilated by the mould. No non-sulphate-sulphur compounds could

be detected in the liquor. Like the mycelial nitrogen, once taken up the sulphur remained in the felt throughout the period of the experiments (20 days).

LITERATURE CITED

- CHUGHTAI, I. D., PEARCE, A. A., WALKER, T. K. (1947). Detection of acetic acid in *A. niger* growing on a glucose medium. *Nature*, **160**, 573.
- HAWK, P. (1938). Volumetric determination of ethereal and inorganic sulfates and total sulfur. *Pract. Physiol. Chemistry*, 11th ed. London: Churchill, 762.
- HOCKENHULL, D. J. D. (1946). Studies in penicillin production by *P. notatum*: 1. A preliminary study of the metabolism of carbon, nitrogen, sulphur, and phosphorus. *Biochem. J.* **40**, 337.
- (1948). Studies in penicillin production by *P. notatum*: 2. Further studies in the metabolism of sulphur. *Ibid.* **43**, 498.
- (1948). Mustard gas mutation in *A. nidulans*. *Nature*, **161**, 100.
- (1949). The sulphur metabolism of mould fungi: the use of 'biochemical mutant' strains of *A. nidulans* in elucidating the biosynthesis of cystine. *Biochem. Biophys. Acta*, **3**, 326.
- RAISTRICK, H. (1931). Studies in the biochemistry of micro-organisms. *Phil. Trans. Roy. Soc., B*, **220**, 1.
- and VINCENT, J. M. (1948). A survey of the fungal metabolism of inorganic sulphates. *Biochem. J.* **43**, 90.

An Examination of a Method of Auxin Assay using the Growth of Isolated Sections of *Avena* Coleoptiles in Test Solutions

JOYCE A. BENTLEY

Department of Botany, University of Manchester

I. A CONSIDERATION OF METHODS OF AUXIN ASSAY

WORK is in progress at Manchester University on problems of the relationship between auxin concentration and morphogenesis of leaves, and also on the possible existence in plants of auxins¹ other than β -indolylacetic acid. In both these studies it is necessary to have accurate methods of auxin assay. This paper presents a consideration of methods of auxin assay and describes attempts to reach a level of efficiency in assay technique suitable for these problems.

Several methods of biological assay of auxins are known. These utilize different types of growth response and different plants (Went and Thimann, 1937; Veldstra, 1944). An examination of data obtained with the same auxin in these different tests shows that its activity varies widely according to the nature of the test material. Consideration will be confined in this paper to methods in which the growth of *Avena* coleoptiles is used, since the response of *Avena* coleoptiles to auxin has been extensively studied and it has been shown that, beyond a length of approximately 1 cm., growth consists entirely of cell elongation (Went and Thimann, 1937).

The curvature method (Went, 1928) is the one that has hitherto been most commonly used in auxin work; it is too well known to require description here. A second method, using coleoptiles split longitudinally, has been investigated by Thimann and Schneider (1939). The inward curvature of the longitudinal strips under the influence of auxins is measured. A third method, which has not been used much in the assay of auxins but which has found wide application in studies on the nature of growth and metabolism, is to measure the increase in length of isolated sections of coleoptiles when placed in solutions containing auxins. This is the method subjected to critical analysis later in the present paper. For the sake of brevity it is proposed here to refer to the method as 'the straight-growth method'. It was first investigated by Bonner (1933) in a study on the relationship between respiration and the action of growth-substances. Other examples of its use are by Schneider (1938), Thimann and Schneider (1938), Kelly (1947), and Thimann and Bonner (1948).

There are several objections to the curvature method as an assay of concen-

¹ The term 'auxin' is used throughout this paper to denote growth-substances active in causing cell enlargement.

tration of auxins. For example, growth on the side of the coleoptile below the agar block is primarily a measure of the amount of auxin diffusing out of the block and its ability to travel down the coleoptile. The rate of diffusion from the block will obviously depend on the molecular weight of the auxin. In the curvature method there is a period of about 90 minutes before a physiological tip is regenerated; after this period the response of a coleoptile cannot be assumed to be due only to auxin diffusing from the block. This period of 90 minutes may be too short for equilibrium to be reached between the block and the coleoptile for all active substances, irrespective of their molecular weights.

It has been shown also that transverse spreading of the auxin across the coleoptile, although slight with β -indolylacetic acid, is quite marked with other auxins (Avery *et al.*, 1937). Methods of assay which depend upon the transport of auxin, if used with due regard to their limitations, are desirable for experiments on the movement of auxins in plants. For experiments on the presence and utilization of auxins *in situ* in various parts of the plant, methods of assay depending on the transport of the active substance are inappropriate.

It is clear, therefore, that for problems on cell elongation a method must be chosen which does not involve the transport of the auxin, but measures only its ability to cause cell elongation. Satisfying this requirement are the split-coleoptile method and the straight-growth method.

The split-coleoptile method does not seem to have been used beyond its first investigation. Preliminary trials carried out by the writer showed that considerable technical difficulties and unreliable results prevented the development of a routine method. The method was therefore rejected.

Preliminary work on the straight-growth method, using techniques followed by previous workers, also gave inconsistent and variable results. But the method is simple to operate, it has wide applications to problems of growth, and on theoretical grounds it is appropriate to the problems being studied in this Department. The remainder of this paper is therefore devoted to an examination of the straight-growth method.

2. AN EXAMINATION OF THE STRAIGHT-GROWTH METHOD USING ISOLATED SECTIONS OF *AVENA* COLEOPTILES IN TEST SOLUTIONS

i. *Experimental procedure*

The standard method developed by Bonner (1933), and elaborated by Thimann and Schneider (1938) and Thimann and Bonner (1948), is briefly as follows.

Oats are husked and allowed to germinate in Petri dishes on moist filter-paper at a temperature of 25° C. They are exposed to red light for the first 48 hours to prevent excessive growth of the mesocotyl. The oats are then allowed to grow in an atmosphere of 85–90 per cent. humidity, the dishes being tilted to facilitate straight growth of the coleoptiles. When they are

between 2–3 cm. long they are ready for use. The first 2–3 mm. of the tip are discarded, and the remainder of the coleoptile is cut, either on a special cutter (Van der Weij, 1932) or by accurately spaced upturned razor blades, into three or usually four cylindrical sections, each of 3 mm. length. These are threaded on toothcombs or glass-rods and placed in the solutions to be tested. All these operations are carried out in red light. The elongation resulting after a period of time up to 60–80 hours is measured by means of a microscope with a micrometer eyepiece.

Under these conditions elongation is proportional to the logarithm of the concentration of the test solution within certain approximate limits: these are the equivalents, in β -indolylacetic acid, of 0.01 mg./l. to between 1.0 and 10.0 mg./l.

ii. *Evaluation of results*

The majority of workers using the straight-growth method express their results as the mean of observations from 30–60 sections per test solution, either as elongation expressed as a percentage of original length, or (if the response of sections in a test solution is being compared with that of controls in water) as the excess of elongation of test sections over the final length of controls, expressed as a percentage of the final length of controls.

Several workers, for example, Bonner (1933) and Thimann and Schneider (1938), remark on the variability of data from this test. They state that variation is such that results can be compared only within any one occasion. Examination of data obtained by other workers confirms this. Thus results obtained on different occasions by Schneider (1938) showed that growth in water varies from 8 to 20 per cent., in 1.0 mg./l. indolylacetic acid (IAA) from 20 to 25 per cent., and in 1 mg./l. IAA and 1 per cent. sucrose from 50 to 85 per cent. Further evidence of the wide variability obtained is shown by the range of points in the graphs given by Kelly (1947).

In the curvature method variation within an occasion is such that Went and Thimann (1937) and Gordon (1946) recommend rejection of values which fall beyond three times the standard deviation of the mean of all values, and recalculation of the mean and standard error without these values. Preliminary results in the present investigation showed that similar variability also existed in the straight-growth method.

It is clear that the straight-growth method cannot be regarded as entirely satisfactory unless the results it gives can be made independent of the occasion on which an experiment is done. In addition, there still remains the variability within occasions, which cannot indefinitely be dealt with merely by rejecting anomalous data. Accordingly a thorough study was made of possible sources of variation. For this purpose the elongation of coleoptile sections in solutions of different concentrations of β -indolylacetic acid, under varying conditions, was examined and the results subjected to statistical analysis where appropriate.

The β -indolylacetic acid was supplied by British Drug Houses Ltd. It was used without any further purification.

3. A CRITICAL EXAMINATION OF POSSIBLE CAUSES OF VARIATION IN THE STRAIGHT-GROWTH METHOD

The following possible causes of variation in response of sections of coleoptiles are considered:

- i. Inconsistency in the method of growing oats.
- ii. Effects of residual auxin in the section on subsequent growth.
- iii. Variation in the length of section used and the position on the coleoptile from which it is cut.
- iv. Variation due to the length of coleoptile from which the section is cut, and the occasion on which the experiment is performed.
- v. The differential response of floating and submerged sections.
- vi. Differences in the duration of the assay.
- vii. Effects of food factors in the solution.

i. *Inconsistency in the method of growing oats*

One advantage of the straight-growth method from the technician's point of view is that a constant humidity chamber is not required; it is possible to handle large numbers of tests in Petri dishes in an incubator at constant temperature. But it is difficult to grow the oats as recommended by previous workers without a constant humidity chamber; in the present investigation it has been found that there is loss of sensitivity and straightness of the coleoptile. The method devised for these studies is to grow the oats on moist filter-paper in Petri dishes until they are 48 hours old. The seedlings are then large enough to handle and are transferred to ignition tubes filled with water and kept in a saturated atmosphere in a beaker or tank. The tubes are just large enough to allow the seeds to fit in the mouth without sinking.

Under these conditions straight coleoptiles are easily obtained and are ready for use (approximately 1.5 cm. long) 76 hours from the time of sowing, at a temperature of 25° C. Coleoptiles of this length are at the point of maximum growth-rate (Went and Thimann, 1937).

The possibility of growing oats in sand has also been investigated. For routine assays this method is desirable, since husking and individual handling of the oats are eliminated. It was found, however, that without a constant humidity chamber there was very variable germination and growth, with loss of sensitivity of the coleoptiles to auxin solutions. Towards the close of the investigation a constant humidity chamber (85 per cent. relative humidity, 26° C.) became available and under these conditions germination and growth of oats in a coarse sand is consistently good. Results of experiments carried out on these oats are given in Table I.

These show that although the sensitivity of sand-grown oats is less than that of water-grown oats, the decrease in response of control sections is of the same order of magnitude as that of sections in solutions of IAA. Under these conditions, therefore, sand-grown oats can be used with confidence.

Sowings are arranged so that tests are always performed at the same time of

day; this minimizes variation in response due to the 24-hour rhythm of growth.

TABLE I

A comparison of the sensitivity of sand-grown and water-grown oats. Results are expressed as percentage elongation, each being the mean of 12 sections, from oats grown under constant conditions of temperature and humidity (see text for details)

Expt. No.	Method of growing	Test solutions (mg./l. I.A.A.)		
		Water	0.1	1.0
1	Water	32	39	62
	Sand	30	34	59
	Difference	2	5	3
2	Water	31	46	57
	Sand	23	38	50
	Difference	8	8	7

ii. Effects of residual auxin in the section on subsequent growth

Residual auxin in the coleoptile greatly affects the sensitivity of the curvature method (Van der Weij, 1931). The desirability of eliminating this source of error in the present investigation was examined by two methods.

The first method is to immerse severed coleoptiles in a solution containing sucrose (Schneider, 1938) for a period of some hours before the test sections are cut from it. The results are given in Expt. 3 (Table II). In this experiment treatment *A* consists of immersion of sections cut direct from a growing coleoptile in a test solution of 0.01 mg./l. IAA. In treatment *B* cut sections

TABLE II

Experiment 3. Analysis of the effect of residual auxin on the response of treated and untreated coleoptile sections. The results are expressed as percentage elongation, each being the mean of 10 sections. The sections were cut from the coleoptile in the order top: middle: bottom

S.D.: standard deviation of the mean.

S.: significant variation at the 1 per cent. probability level.

Treatment	Position on coleoptile					
	Top		Middle		Bottom	
	% elongation	S.D.	% elongation	S.D.	% elongation	S.D.
A (control)	21.6	1.1	17.4	1.5	8.9	1.1
B (pre-treated)	13.6	1.6	15.0	1.9	13.9	1.8

Analysis of variance

Cause of variance	Degrees of freedom	Mean square	Variance ratio (F)	Significance
Treatment	1	46.64	1.92	—
Position	2	265.63	10.8	S
Interaction	2	164.61	6.8	S
Error	54	24.4	—	—

were soaked for 4 hours in 1 per cent. sucrose with 0.01N KH_2PO_4 before immersion in the test solution. The sections were approximately 3 mm. long, and were tested in the order in which they occurred on the coleoptile for the purposes of another experiment (section iii). Because of errors in cutting sections of this size they were all measured before testing as well as after.

It is seen from Table II that there is no significant variation between treatments *A* and *B*. The results for different parts of the coleoptile are considered on p. 207. Since variation due to this is eliminated by other means, and since obviously the sensitivity of the test is not increased by pre-treatment of the coleoptile, it was decided to reject this method.

The second method involves decapitation of the growing coleoptile and lapse of an interval of time before use.

The length of the interval of time between decapitation and placement of agar cubes (termed IDP) is important in the curvature method (Schneider and Went, 1938), but has been shown by Schneider (1938) to have little effect on the response of the coleoptile in the straight-growth method. This was confirmed by experiments, the results of which are given in Table III.

TABLE III

The effect of IDP in the straight-growth method. The results are expressed as percentage elongation, each being the mean of 12 sections of initial length 10 mm. measured after 24-hour test

Expt.	IDP (min.)	Test solutions (mg./l. IAA)							
		Water		0.01		0.1		1.0	
		% elong.	S.D.	% elong.	S.D.	% elong.	S.D.	% elong.	S.D.
4	0	17	1.3	23	2.0	44	3.8	62	7.7
4	90	15	1.7	32	3.8	56	3.8	47	5.6
5	0	15	2.4	22	2.1	48	4.3	53	3.8
5	90	16	2.1	20	1.8	34	4.6	32	5.8

These results indicate that residual auxin neither increases variability nor decreases sensitivity. Therefore no advantage is gained by an initial decapitation.

The fall in sensitivity to high concentrations of auxin, of oats having an IDP of 90 minutes, and the fact that growth of controls appears to be independent of IDP (reported also by Schneider (1938) for the curvature method), are interesting phenomena which need further examination. In any event, the fact that IDP is so important in the curvature method and not in the straight-growth method requires explanation.

From the data it was decided that there was no need for pre-treatment of the coleoptile.

iii. *Variation in the length of section used and the position on the coleoptile from which it is cut*

A. The response of 3-mm. sections. The majority of workers use 3-mm. sections, and cut as many as three to four from each coleoptile. Schneider

(1938), in his results, separates response of sections according to position on the coleoptile, showing a range of from 58 to 86 per cent. elongation from the bottom to the top of the coleoptile in 1 per cent. sucrose and 1 mg./l. IAA. Data are not available to test whether, within the proportionality range 0.01 mg./l. to 1.0 mg./l. IAA, this variability would interfere with variation due to different concentrations of the acid, but the results of Expt. 3 (p. 205), in which variation due to position of the section on the coleoptile is seen to be highly significant, suggest that this might be so.

Further experiments have confirmed this impression, and show that it is unsatisfactory to compare sections except those taken from the same position on the coleoptile. Moreover, experiments have repeatedly shown that percentage elongation is less with 3-mm. sections than with 10-mm. sections. This unexpected conclusion might be explained on the assumption that cells at each end of the section are damaged, and do not react.

B. *The response of 10-mm. sections.* Preliminary experiments on 10-mm. sections confirmed the reports of previous workers that growth in indolyl-acetic acid solutions is proportional to the logarithm of the concentration within the limits 0.01 to 1.0 mg./l. Below 0.01 mg./l. the test is insufficiently sensitive, and above 1 mg./l. response is obscured by partial inhibition.

The response of 10-mm. sections is investigated further in section iv (p. 208).

C. *The measurement of sections.* Sections 3 mm. long are usually measured with a microscope with a micrometer eyepiece. This is not practicable for 10-mm. sections. However, the greater precision of measurements of 3-mm. sections is not justified, because errors of measurement, especially if there is a slight difference in response of the two sides of the coleoptile, can be as much as 10 per cent. of the total length, and errors of cutting are of the same order.

The possibility of error in the measurement of 10-mm. sections was examined as follows. Sections were measured with a dissecting microscope, magnification $\times 10$, using a transparent rule accurate to the nearest 0.5 mm., as scale. The sections were placed on the rule and the rule moved across the field of vision, readings always being taken in the centre of the field of the eyepiece. Lengths could be read off directly. The same sections were then measured with a binocular microscope, magnification $\times 50$, using a mechanical stage accurate to the nearest 0.1 mm. This involved considerable manipulation of the mechanical stage and the recording of two readings for each section.

The error between the two methods was approximately 1 per cent.

The ability to obtain accurate measurements by different operators was tested as follows. Two microscopes were used, a binocular dissecting microscope, magnification $\times 17$, and a binocular microscope, magnification $\times 50$. Sections were measured by two operators on both microscopes. The results showed that between operators, and between microscopes of varying magnification, the error is only of the order of 1–2 per cent.

The use of 10-mm. sections measured with a dissecting microscope with a transparent rule therefore seems to offer rapid and reliable results.

iv. *Variation due to length of the coleoptile from which the section is cut, and the occasion on which the experiment is performed*

(a) *Length of coleoptile.* It is difficult in routine sowings of oats to obtain such uniformity of size of seed, and speed of germination within a batch, that all seedlings of the same time-age have coleoptiles of the same length. Within a batch 76 hours old there may be variation in length from approximately 1 to 2 cm.

The importance of length of coleoptile rather than age for the study of physiological problems has been noted by several workers. Preston (1944), when studying wall-deposition in *Avena* coleoptiles, has expressed all his findings in terms of coleoptile length rather than time-age, the former being considered a 'variable of greater physiological importance'.

In the light of these considerations it was decided to study the response of sections cut from coleoptiles sown at the same time under identical conditions of growth, but of different lengths.

(b) *Occasion.* An analysis of variance on preliminary experiments showed that variation due to different occasions is highly significant. The experiment on the effect of length of coleoptile was repeated on a number of occasions to test whether, under these stringent conditions of selection of material, variation from occasion to occasion still occurred.

The results of three of these experiments are summarized in Fig. 1, and Tables IV and V.

The conclusions to be drawn from these results will now be examined.

(i) From Fig. 1 it is clear that there is a consistent response to concentrations of IAA; also that the response is similar, though not identical, on three occasions.

(ii) From Table V the following points emerge:

1. There are highly significant differences in response due to concentration of IAA.
2. There is still significantly more variation between occasions than among replicates on any individual day.
3. There is a significant effect due to length of coleoptile from which the section is cut.
4. If the variation due to occasion is used as error, that due to concentration of IAA is still significant at the 5 per cent. probability level, so that even though differences in response with time still exist, they are not large enough to obscure differences due to concentration of auxin.
5. There is a very large interaction between occasion and length of coleoptile from which the section is cut. This means that the effect of length depends very much on the occasion, suggesting that the conditions of germination such as rate of imbibition of water, and mobilization of enzymes, which must ultimately determine the type of coleoptile produced, are the factors responsible for fluctuations in response from time to time.

A question which needs further study is whether the variation in these

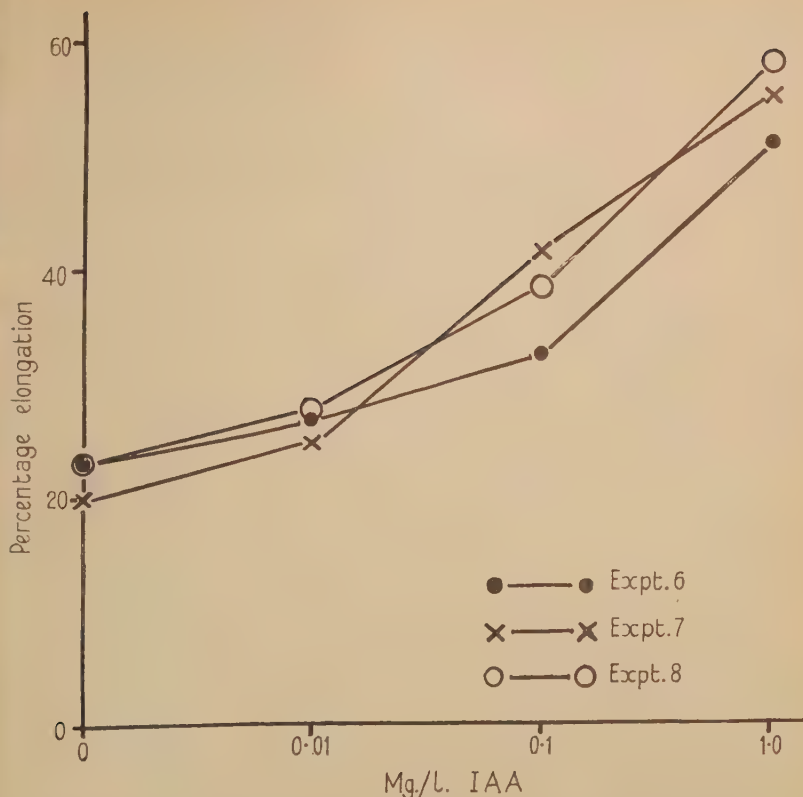


FIG. 1. Percentage elongation of coleoptile sections of initial length 10 mm. in solutions of IAA. Each point is the mean of 30 sections cut from coleoptiles 1.3 to 2.3 cm. long.

TABLE IV

The effect of coleoptile length on response. L (Long): 2.0 to 2.5 cm.; M (Medium): 1.5 to 2.0 cm.; S (Short): 1.3 to 1.5 cm. All figures are the means of 10 sections of original length 10 mm. expressed as percentage elongation

Expt. No.	Coleoptile length	Conc. IAA in mg./l.			
		0	0.01	0.1	1.0
6	L	21	25	29	42
	M	24	28	31	49
	S	24	29	40	59
7	L	19	21	40	51
	M	21	27	42	55
	S	20	28	44	61
8	L	19	24	34	54
	M	24	30	37	60
	S	26	30	45	64

factors is inherent in the oat seedling, or whether it could be eliminated by such precautions as selection of seeds of uniform weight and use of detergents to ensure uniform wetting of the grain.

TABLE V

Analysis of variance on the effects of occasion, concentration of IAA, and length of coleoptile, on the response of 10-mm. sections

D.F., degrees of freedom; F, variance ratio; S, significance.

XX, significance at the 1% probability level.

X, " " " " 5% " "

Cause of variance	D.F.	Mean square	F.	S.	F/occasion	S
Occasion	2	529	6.94	XX	—	—
Concentration of IAA	3	21,530	282.00	XX	40.70	X
Length	2	1,411	18.50	XX	—	—
Occasion $\times$ IAA conc.	6	1,212	15.90	XX	—	—
Occasion $\times$ length	4	17,259	227.00	XX	—	—
Length $\times$ IAA conc.	6	918	12.10	XX	—	—
Triple interaction	12	440	5.80	—	—	—
Error	366	76	—	—	—	—

6. The semi-fiducial interval of the figures in Table IV is 3.95. Therefore, reference to the table shows that differences in concentrations of indolyl-acetic acid can be detected with a precision of 95 per cent., as follows:—between 0 and 0.1 on all occasions; between 0.1 and 1.0 on all occasions; between 0.01 and 0.1 on all but three occasions; and between 0 and 0.01 on only one occasion. A concentration of 0.01 mg./l. IAA is therefore just below the lower limit of sensitivity.

It is noteworthy that the most highly significant differences occur when the shortest coleoptiles are used for assay.

The two points of major importance for the purpose of assay of unknown auxins are: (i) significant differences due to concentrations corresponding to the concentrations of IAA given above (para. 6) are obtainable between experiments in spite of variations due to occasion. We are therefore justified in superposing the curves for separate occasions by 'calibrating' the response on any one occasion with a known amount of IAA. (ii) coleoptiles of uniform length should be selected for use within any one assay.

(v) *The differential response of floating and submerged sections*

Bonner (1933) states that differential access of oxygen has no effect on response, although he claims that the action of auxin is connected with respiration (Bonner, 1933, 1933*a*, 1936). He concludes that the sections need not float on the solution.

In a study of growth in isolated coleoptile sections under varying conditions, Thimann and Bonner (1948) report experiments which show that better growth is obtained in floating sections in the presence of auxin. In the absence of auxin there is no difference between floating and submerged sections.

In numerous experiments the writer has observed that there is better growth

in floating sections. While this may not necessarily be related to availability of oxygen at the seat of auxin action, it has great practical importance in reducing variability in the test. It is recommended that sections should be mounted on glass capillary tubes sealed to ensure that they float.

vi. *Differences in the duration of the assay*

In the absence of a food factor (see section vii) it was found that the maximum response was obtained after 20–24 hours at a temperature of 25° C. A longer period gave no further response unless the conditions were changed.

vii. *Effects of food factors in the test solution*

Schneider (1938) and Thimann and Schneider (1938) report that maximum growth is obtained in solutions containing 1 per cent. sucrose and 0.01N potassium chloride, in addition to the growth-substance. The writer has carried out numerous experiments with solutions containing these two constituents, both together and separately, with very variable results.

The sucrose was tested for inhibitors, and found to be free from them. It was therefore used without any further purification. The potassium chloride was Analar standard. In all the experiments the concentrations used were 1 per cent. sucrose and 0.01N potassium chloride, and the test was of 48 hours' duration to allow completion of growth in the presence of sucrose. It is not necessary to tabulate all the data; briefly the results were as follows:

i. There is evidence that KCl inhibits elongation. This has been found in experiments comparing elongation in water with that in KCl in addition to experiments using IAA. In all cases there is lesser elongation in KCl. This inhibition has been observed by other workers, and has been interpreted (Frey-Wyssling, 1948, p. 179 and fig. 130*b*) as due to competition between the KCl and the —OH groups in the cellulose lattice of the cell-wall for water molecules. This prevents separation of the cellulose micelles by weakening of the subsidiary valency bonds connecting adjacent —OH groups, and so leads to an inhibition of growth. If this explanation is correct, then alkaline or alkaline-earth salts should not be used as accessory food factors.

ii. There was a disquieting amount of variability in response to sucrose. Some experiments gave responses with sucrose alone as high as is given by 1 mg./l. IAA alone; in other experiments there was no marked increase in elongation when sucrose was used, except with IAA. This is a strong argument against its use as a food factor in assays.

iii. Experiments using 1 per cent. glucose as a food factor gave good growth, but the growth of controls without IAA was correspondingly increased, so that when results were expressed as excess elongation of sections in auxin solutions over controls in water, no better results were obtained than when no food factor was used.

On all these grounds it was decided not to introduce a food factor into the test solutions.

This completes the examination of the possible causes of variation in the

straight-growth method and enables a revised experimental procedure to be detailed.

4. A REVISED EXPERIMENTAL PROCEDURE FOR THE STRAIGHT-GROWTH METHOD

Oats are husked and allowed to germinate on moist filter-paper in Petri dishes for 48 hours at a temperature of 25°C . They are exposed during this period to red light to suppress growth of the mesocotyl. If a constant humidity chamber is not available, the seedlings are transferred after 48 hours to ignition tubes filled with water and kept in a saturated atmosphere in a beaker or tank. If a constant humidity chamber is available, they need not be disturbed until ready for use. All further growth is in the dark. An alternative method is to grow oats, without husking, in coarse sand at 85 per cent. humidity and 25°C ., and to expose them after their appearance to red light for 48 hours. After 76 hours from the time of sowing, the coleoptiles are approximately 1.5 cm. long and are ready for use.

Coleoptiles of uniform length are selected, severed from their mesocotyls, and laid in batches of ten on a wet glass slide, the tips exactly to the edge of the slide. A 10-mm. section is then cut from each of them by means of two accurately spaced upturned razor blades. A guard, exactly 3 mm. from one of the blades, ensures that a 3-mm. tip is cut from each coleoptile and rejected. The sections are threaded on sealed glass capillaries and floated on the test solutions. For the problems under consideration in this Department it is desired to work with small volumes of solutions. Therefore twelve sections in a volume of 10 ml. in a small Petri dish are used for each test. This could be varied according to the nature of the problem. All these operations are performed in phototropically inactive light ($> 550\text{ m}\mu$).

The sections are allowed to elongate for 24 hours at a temperature of 25°C . They are then measured with a dissecting microscope, using a rule accurate to the nearest 0.5 mm.

As controls, the response of sections in distilled water and in 1 mg./l. IAA is tested on each occasion. Results can be calculated as the excess elongation of test sections over the final length of controls in water, expressed as a percentage of the final length of controls, or in terms of indolylacetic acid.

5. SUMMARY

1. Methods of auxin assay using the *Avena* coleoptile are discussed.
2. A review is given of experimental procedure and evaluation of results in the straight-growth method using isolated sections of coleoptiles in test solutions.
3. Possible sources of variation in the straight-growth method are investigated and discussed.
4. A revised experimental procedure for the straight-growth method is described.

The author wishes to thank Professor E. Ashby for his advice and encouragement during the course of the experiments.

Thanks are also due to Mr. D. Payne, a technical assistant in the Botany Department, for his assistance in some of the assays.

Note. Since the preparation of this work for publication an interesting paper by C. M. Larsen ('Hereditary Variations in the Sensitivity of *Avena* Coleoptiles to Growth Substance', *Physiologia Plantarum*, **1**, 265-77 (1948)) has become available. He demonstrates the existence of three physiologically different strains in the so-called pure line of 'Victory' oats used in *Avena* tests. This may account for the differential growth rates of the germinating oats mentioned in section 3 (iv). Further selection of test material would therefore achieve the same reduction of variability within any one occasion as strict selection of coleoptile length at the time of testing. It is improbable, however, that it would affect variation between occasions.

LITERATURE CITED

- AVERY, G. S. JR., BURKHOLDER, P. R., CREIGHTON, H. B. (1937). *Avena* coleoptile curvature in relation to different concentrations of certain synthetic substances. *Amer. J. Bot.* **24**, 226-32.
 BONNER, J. (1933). The action of the plant-growth hormone. *J. Gen. Physiol.* **17**, 63-76.
 — (1933a). Studies on the growth-hormone of plants. IV. On the mechanism of the action. *Proc. Nat. Acad. Sci.* **19**, 717-19.
 — (1936). The growth and respiration of the *Avena* coleoptile. *J. Gen. Physiol.* **20**, 1-11.
 BOYSEN-JENSEN, P. (1936). *Growth-hormones in plants*. Translated and revised by G. S. Avery, Jr. and P. R. Burkholder. New York, 1936.
 FREY-WYSSLING, A. (1948). *Sub-microscopic morphology of protoplasm and its derivatives*. New York, Amsterdam, 1948.
 GORDON, S. A. (1946). Auxin-protein complexes of the wheat grain. *Amer. J. Bot.* **33**, 160-9.
 KELLY, S. (1947). The relationship between respiration and water-uptake in the oat coleoptile. *Ibid.* **34**, 521-6.
 PRESTON, R. D., and CLARK, C. S. (1944). Wall structure and growth. II. Wall deposition in the *Avena* coleoptile. *Proc. Leeds. Phil. Soc.* **4**, 201-14.
 SCHNEIDER, C. L. (1938). The interdependence of auxin and sugar for growth. *Amer. J. Bot.* **25**, 258-70.
 — and WENT, F. W. (1938). A photokymograph for the analysis of the *Avena* test. *Bot. Gaz.* **99**, 470-95.
 THIMANN, K. V., and SCHNEIDER, C. L. (1938). The role of salts, hydrogen-ion concentration, and agar in the response of the *Avena* coleoptile to auxins. *Amer. J. Bot.* **25**, 270-80.
 — (1939). Differential growth in plant-tissues. II. A modified auxin test of high sensitivity. *Ibid.* **26**, 792-7.
 — and BONNER, W. D. (1948). Experiments on the growth and inhibition of isolated plant parts. I. The action of iodo acetate and organic acids on the *Avena* coleoptile. *Ibid.* **35**, 271-80.
 VELDSTRA, H. (1944). Researches on plant growth substances. IV. The relation between chemical structure and physiological activity I and II. *Enzymologia*, **11**, 97-137, 137-63.
 WEIJ, H. G. VAN DER (1931). Die quantitative Arbeitsmethode mit Wuchsstoffe. *Proc. Kon. Akad. Wetensch. Amsterdam*, **34**, 875-92.
 — (1932). Der Mechanismus des Wuchsstofftransportes. *Rec. Trav. Bot. Neerl.* **29**, 379-496.
 WENT, F. W., and THIMANN, K. V. (1937). *Phytohormones*. New York.
 — (1928). Wuchsstoff und Wachstum. *Rec. Trav. Bot. Neerl.* **25**, 1-116.

The Fine Structure of Bamboo Fibres

I. OPTICAL PROPERTIES AND X-RAY DATA

R. D. PRESTON AND K. SINGH¹

Department of Botany, University of Leeds

WITH 1 PLATE

INTRODUCTION

It is a number of years since it was first suggested that in many plant fibres the cell wall is heterogeneous as regards cellulose chain orientation (see e.g. Steinbrinck, 1927; Reimers, 1922), but although reference is made to this conception from time to time, the lack of any evidence from strictly physical determinations, and particularly the lack of any sign of heterogeneity in X-ray diagrams, has tended to keep it very much in the background. Now that it has been shown, however, by unequivocal optical determinations on undistorted tissues, that in conifer tracheides the view of Bailey and his co-workers (Bailey and Kerr, 1935; Bailey and Vestal, 1937) that an outer layer exists in the wall in which the molecular chains of cellulose lie along a spiral flatter than that in the rest of the wall, and that of Preston (1939, 1946) that the angular dispersion is different, have both been found correct (Wardrop and Preston, 1948), it seems desirable to re-examine the fibres from this point of view. This re-examination has already been commenced with sisal fibres, where it has appeared, again without the slightest shadow of doubt, that essentially the same type of structure occurs (Preston and Middlebrook, 1949). The present paper deals with the fibres of bamboo from a similar point of view, although the methods employed are quite different from those used for wood tracheides and for sisal. In wood tracheides advantage was taken of the fact that the cells are arranged in regular radial seriation; in sisal the slow growth of the fibre enabled the outer layer to be investigated as an individual unit; and the lack of any corresponding attributes makes the bamboo problem much more formidable. Here the solution lies in the rigorously accurate determination of the optical properties of the fibres in longitudinal and transverse section.

Bamboo fibres were chosen for two main reasons. Firstly, because developing, as they do, from daughter cells produced by longitudinal division of cells which had already become parenchymatous, they form a striking contrast to wood cells, which develop directly from a lateral meristem, and to the other monocotyledonous fibre sisal, which develops directly from the meristem at the base of the leaf. Secondly, because the increasing attention being paid in India and elsewhere to bamboo as a source of cellulose for industrial purposes makes it imperative that the fine structure should be known in as much detail as possible. In view of the considerable difference between the origin of bamboo

¹ An officer of the Cellulose and Paper Pulp Division, Forest Research Institute, Dehra Dun, India.

fibres and of conifer tracheides and of the very marked variation in fibre length, the opportunity has also been taken to see if a relation of fibre dimensions to wall organization occurs such as has already been found in tracheides (Preston, 1934, 1948; Wardrop and Preston, 1948). These points will be discussed in the present paper. This is done partly in an attempt to determine if the important relations discovered for conifer tracheides, and suggested also for sisal fibres, is in fact a phenomenon general to elongated cells and partly because, if the relation is found to hold, then this would be of considerable importance in the technology of bamboo; for it would imply a relation also between other physical properties and fibre length, e.g. an increasing tensile strength and Young's Modulus with increasing length. Further work on the interrelationships of birefringence, density, and fibre length, and on some 'abnormal' reflections in the X-ray diagram of bamboo will be dealt with separately in later papers in this series.

In the present paper, then, attention is given largely to the general molecular architecture of bamboo fibres and particularly the relation of molecular structure to fibre dimensions.

MATERIAL AND METHODS

The material available consisted in the main of mature stems of *Dendrocalamus strictus*, *D. longispathus*, *Melocanna bambusoide*, and *Bambusa arundicea*, provided by the courtesy of Mr. D. Stuart, then President of the Forest Research Institute, Dehra Dun, India, to whom our thanks are due. Sections were cut of these species without further treatment, and individual fibres were isolated by normal maceration techniques to be described. Major extinction positions (m.e.p.s) were made as usual on single cell walls, since it was anticipated that the cellulose chains would run in spirals round the cell. In view, however, of the unusually thick walls and correspondingly narrow lumina of many of these fibres, the technique usually employed in this laboratory to obtain single cell walls (Preston, 1934) could not be used. The material was therefore cut longitudinally in sections 5–10 μ thick (and therefore thinner than a single cell diameter) and the sections were subsequently macerated by alternate treatments with cold chlorine water and hot 3 per cent. sodium sulphite. The separated cells were then attached to microscope slides by albumen fixative, dehydrated, and mounted in Canada balsam. Determinations were made only on parts of cells in which it was certain that only one cell wall remained.

Birefringences in transverse sections were determined from the phase difference measured on thin sections, using a de Sénarmont compensator, and the section thickness (Preston, 1947). It follows that the figure given for each sample is therefore an average over the sample. An equivalent method could not be used for determination of birefringences in longitudinal view in virtue of the cylindrical form of the fibres, and therefore the two refractive indices were determined separately by an immersion method. The procedure adopted

was as follows. In order to avoid subjective errors in unconsciously choosing longer fibres for determination, and for other reasons which will appear later (p. 219), fibres were selected, by hand, having lengths of 1 mm., 2 mm., 3 mm., 4 mm., and 5 mm., giving five fractions of exactly known fibre length. The refractive indices of these fractions were determined by a modification of a method described by Hermans (1946). The fibres were placed in a capillary tube (Fig. 1) on one end of which two bulbs were blown, and the other end sealed. Immersion liquid was introduced into bulb A and P_2O_5 into bulb C,

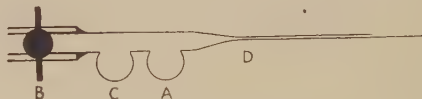


FIG. 1. For explanation, see text.

and the tube was then highly evacuated and sealed. The capillary tube was then heated at 105°C . for 20 hours, keeping the bulbs cool and, after cooling, the immersion liquid was run into the capillary tube and driven in by breaking the seal at B beyond the bulbs. The capillary tube was then sealed off at D, mounted in α -monobromnaphthalene, and examined under the polarizing microscope. Immersion liquids of different refractive indices were prepared by mixing α -monobromnaphthalene (n_D 20°C . = 1.658, b.p. 279°C .) and liquid paraffin (n_D 20°C . = 1.481, b.p. about 270°C .). These mixtures have a low-temperature coefficient of refractive index (-0.0004 per $^{\circ}\text{C}$.), have almost equal volatility, and do not swell the dried fibres. The average birefringence could then be determined from a curve relating birefringence to cell length by reading off the value at the average cell length.

X-ray photographs were usually taken on a Shearer type tube using CuK_{α} radiation collimated by a slit 0.5 mm. diameter and using a flat film. For clearer resolution of the centre of the photograph, in special circumstances recorded later, a Matchlett tube was used with a finer slit (0.25 mm. diameter). All diagrams were calibrated from the diagram of silver. Specimens were normally prepared by cutting bamboo parallel to fibre length into pieces 1 mm. thick. These were arranged in the spectrometer with the X-ray beam normal to cell length. With a slit diameter, therefore, of 0.5 mm., the specimen studied consisted of a cylinder 1 mm. long and about 0.5 mm. diameter and containing parts of about 800 fibres.

Finally such chemical analyses as were made were carried out by normal methods.

RESULTS

A. Proximal analysis

Chemical analyses were carried out on one species only—*Dendrocalamus longispathus*—since only a rough estimate of the relative amounts of the various substances present was needed. The analysis was carried out on

material ground to pass a 70-mesh sieve. The results were as follows, all the figures being based on dry weight:

Water	7.71%
Benzene-alcohol soluble	1.45%
Holocellulose (containing all pentoses)	66.5 %
Lignin	28.3 %
Xylan	18.37%
Glucuronides	4.2 %
Cross and Bevan cellulose	60.8 %

The fibres may therefore be classed as high lignin, high xylan fibres. Silver staining of thin transverse sections by the method of Coppick and Fowler (1939) shows a rather unusual distribution of lignin in that *inner* layers are apparently the more heavily lignified.

B. Optical determinations

The results of m.e.p. determinations on whole single walls are given in Table I, where it is clear that, in so far as the m.e.p. gives a true picture of cellulose chain organization (see below) the fibres are wound with a molecular spiral in which the chains are inclined to cell length at an average angle of

TABLE I
Cell length and spiral angle in bamboo fibres

Species	Angle, θ , between fibre length and m.e.p. (degrees)		Cot θ	Fibre length (mm.)
	Range	Average		
<i>D. longispathus</i>	2.0—8.4	5.00 ± 0.24	11.43	3.04 ± 0.11
<i>B. arundinacea</i>	2.0—7.5	5.40 ± 0.26	10.58	2.86 ± 0.09
<i>D. strictus</i>	3.0—7.3	6.44 ± 0.34	8.91	2.74 ± 0.10
<i>M. bambusoide</i>	6.5—14.0	10.40 ± 0.38	5.45	1.83 ± 0.07

5–6°, except in the case of *Melocanna bambusoide*, where the angle is about 10°. It is clear from the table that this variation in direction of m.e.p. is closely related to differences in fibre length in that the longer fibres are wound with steeper spirals much as reported by Preston (1934, 1948) for conifer tracheides. This is shown more clearly in Fig. 2, where the line drawn through the points (not calculated, since the points are so few) represents the relation

$$L = 750 + 200 \cot \theta,$$

where θ is the average inclination to be expected in fibres of average length L (in μ). While the points are too few for any reliance to be placed on a quantitative relation of this kind, it is nevertheless satisfactory that the relation is of the form described for tracheides (Preston, 1948) and the constants are of the same order.

This relatively steep orientation of the bulk of the chains in the wall is fully supported by the brightness between crossed nicols of fibres in longitudinal view and by the fact that in transverse section the bulk of the wall is dark. Nevertheless the wall is undoubtedly more complicated than such a simple

picture would suggest, for, in transverse section, layers can be readily observed which differ from the rest of the wall in being bright (Plate I, Fig. 1 (b)). Thus

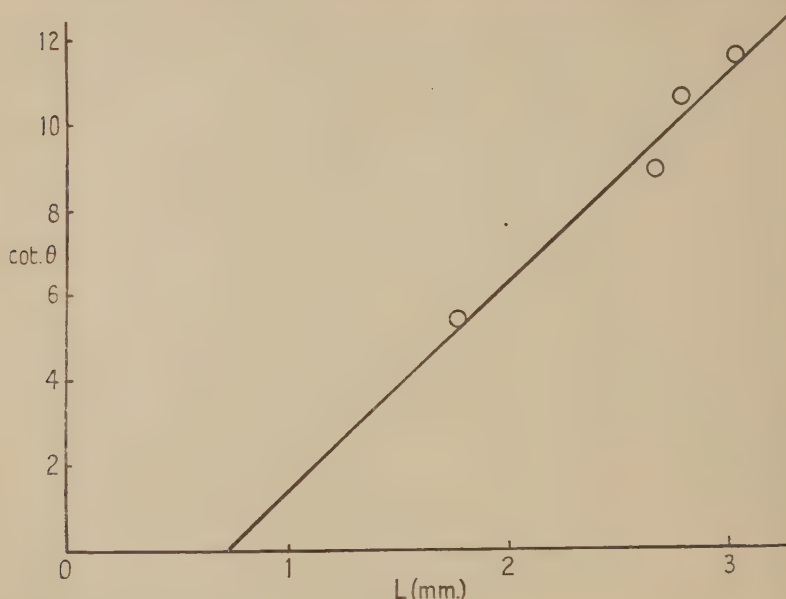


FIG. 2. For explanation, see text.

the outermost lamella is always bright and often a number of thin intermediate lamellae lying in the common dark background are almost equally bright so that bamboo resembles in structure the wood fibres of *Pandanus*. It remains,

TABLE II

The birefringence in transverse section of the different wall layers of bamboo fibres. Each mean corresponds to 25 observations. The errors shown are standard errors

Species	Treatment	Section thickness	Wall* layer	Phase difference	Birefringence	θ
<i>D. strictus</i>	Untreated	4.56 μ	1	65.2 $\pm$ 13.19	0.023 $\pm$ 0.005	34.5 $\pm$ 4.0
			2	17.2 $\pm$ 9.9	0.0062 $\pm$ 0.004	14.0 $\pm$ 8.0
			3	9.0 $\pm$ 5.7	0.0032 $\pm$ 0.002	7.0 $\pm$ 5.0
	Delignified	6.27 μ	1	93.2 $\pm$ 22.7	0.024 $\pm$ 0.006	35.0 $\pm$ 5.0
			2	38.8 $\pm$ 16.3	0.010 $\pm$ 0.004	20.5 $\pm$ 5.5
			3	20.0 $\pm$ 4.0	0.005 $\pm$ 0.001	12.0 $\pm$ 2.0
<i>D. longispathus</i>	Untreated	7.39 μ	1	102.8 $\pm$ 12.1	0.023 $\pm$ 0.003	35.0 $\pm$ 4.0
			2	—	—	—
			3	—	—	—
	Delignified	7.60 μ	1	108.2 $\pm$ 10.0	0.023 $\pm$ 0.002	34.0 $\pm$ 1.5
			2	43.0 $\pm$ 7.0	0.009 $\pm$ 0.002	19.0 $\pm$ 2.0
			3	19.0 $\pm$ 3.3	0.004 $\pm$ 0.001	10.0 $\pm$ 2.0

* 1 = outer bright layer; 2 = middle bright layer; 3 = inner bright layer.

therefore, to determine the conditions in these bright layers. This will be attempted later in this paper. For the moment we note that the birefringence in transverse section, determined as described above, is the same (0.023) in

TABLE III

Refractive indices of fibres of *D. strictus* in longitudinal view
Temperature, 25° C. Na_D light

Length (mm.)	$n_v^{\parallel}$	$n_{\alpha}^{\parallel}$	$n_v^{\parallel} - n_{\alpha}^{\parallel}$	θ	$\text{Cot } \theta$
1	1.569	1.530	0.039	40° 44'	1.1612
2	1.573	1.529	0.044	37° 44'	1.3001
3	1.576	1.528	0.048	34° 53'	1.4344
4	1.580	1.527	0.053	31° 34'	1.6276
5	1.584	1.527	0.057	28° 4'	1.8754

the outer lamellae of *Dendrocalamus strictus* and *D. longispathus* (Table II). In both species the birefringence of the other bright layers steadily decreases as the lumen is approached, and here the value increases on delignification.

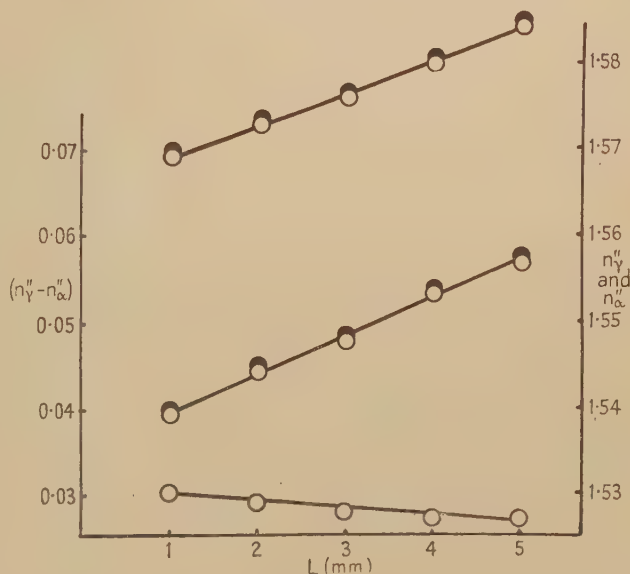


FIG. 3. The relation between fibre length and refractive indices. Open circles: *Dendrocalamus strictus* (Table III). solid circles: Corresponding set of data for *D. longispathus*.

The absence of any effect of delignification on the outer layer is probably associated with the low degree of lignification in that region. In longitudinal view, both the major refractive index ($n_v^{\parallel}$, parallel to cell length) and the birefringence, measured by the immersion method described above (and therefore referring to the outer layer only), increase linearly as cell length increases

(Table III, Fig. 3). The minor refractive index ($n_{\alpha}^{\parallel}$ for light vibrating perpendicularly to cell length) decreases as cell length increases. Undoubtedly, therefore, some structural feature in this outer layer is associated with cell length much as was recorded above for the m.e.p. of the whole wall thickness. From the relations illustrated in Fig. 3 and using the average fibre lengths recorded in Table I, the average refractive indices and birefringence can be read off with some accuracy. These will be used below in an attempt to elucidate the structure of the outer lamella.

C. The X-ray diagram

The deduction from optical determinations that the bulk of the cellulosic component of the material consists of chains oriented almost parallel to cell length is confirmed by the X-ray diagrams, a typical example of which is given in Plate I, Fig. 2. Here again *Melocanna bambusoides* differs somewhat from the rest of the species studied in that the lateral arcs are much more spread, and therefore the orientation parallel to cell length is less perfect.

Clearly it is impossible from such photographs alone to determine the preferred orientation in a single wall; for either of two possible structures might be expected to give diagrams of this type:

- (a) The chains might lie on an average longitudinally but with some angular dispersion (more marked in *M. bambusoides*) about this mean direction.
- (b) They might be arranged in a spiral with sufficient angular dispersion about the spiral direction to remove the higher intensity one might otherwise expect at the ends of each arc (Preston, 1946; Astbury, Preston, and Ranganathan, 1950). In this case the spiral might be flatter in *M. bambusoides*.

Our experience with fibres suggests this latter condition to be correct, and this would be in agreement with the optical findings. This being so, there is no point in determining the 'orientation' factor used by Hermans (1946) as a criterion of structure, for this clearly has no precise meaning in spirally wound fibres, as was in fact pointed out long ago by Sisson (1933). From earlier work in this laboratory on model spirals (Astbury, Preston, and Ranganathan, 1950) it may be seen that if the fibre is indeed wound with a molecular spiral then a rough approximation to the spiral angle may be made from the angle ϕ between the most intense point of the 002, 101, and 10 $\bar{1}$ arcs and the point of most rapid decrease in intensity along the same arc, from the relation

$$\sin S = \cos \theta \cos \phi,$$

where θ is the glancing angle and S is the angle between the spiral winding and the longitudinal cell axis. Using this relationship and the spread of the arcs measured photometrically, the spiral angle turns out to be 9–10°. This compares satisfactorily with the m.e.p. determinations quoted above. With the other species which were not determined photometrically, a rough visual estimate gives the values presented in Table IV, where a rough correlation is again detectable between spiral angle and fibre length.

It is therefore confirmed that the bulk of the cellulose chains in the wall are oriented in steep spirals. It is clear also that the X-ray diagrams are in harmony with the relation between fibre length and wall structure suggested above on

TABLE IV

Degree of orientation of bamboo fibres as expressed by the half-intensity width of the 002 arcs

Species	2β	Length (mm.)
<i>Neuhouzea Dullooa</i>	20	—
<i>B. polymorpha</i>	20	3.59
<i>D. longispathus</i>	20	3.04
<i>B. Arundinacea</i>	21	2.86
<i>D. strictus</i>	21	2.74
<i>M. bambusoide</i> , mature	49	1.83
" 3-6 months	33	—
<i>D. strictus</i> , macerated fibres	23	2.0
" " "	21	3.0
" " "	17	4.0

the basis of optical evidence. During the course of this investigation the opportunity was taken of testing this point within a single species. Macerated fibres of *Dendrocalamus strictus* of uniform length were selected and arranged parallel by hand under the microscope. The resulting fibre bundle was embedded in gelatine and the gelatine block placed in an X-ray spectrometer in the usual way. The angular spreads of the 002 differentiation arcs are included in Table IV for fibres of 2-mm. to 4-mm. length, and it is again clear that in longer fibres the chains of cellulose are in fact more closely parallel to cell length.

In addition to the arcs accepted as typical of cellulose, other arcs of longer spacing were commonly found to be present in all these photographs of bamboo fibres. These will be discussed in a later paper, but it may be said here that some of these arcs are due to the presence of wax.

Evidence from swelling behaviour

It has frequently been reported in the literature that fibres behave on swelling as though enveloped in an outer restricting sheath, and bamboo fibres are no exception. When transverse sections are treated with NaOH or H₂SO₄, the cells swell to an extent depending on the concentration of the reagent (Fig. 4), but the percentage increase in wall thickness is always greater than in cell diameter. In sulphuric acid no swelling occurs below 63 per cent.; in stronger solutions the walls swell suddenly and become disorganized. This behaviour, in terms of earlier work on hemp (Kundu and Preston, 1940) and jute (Preston, 1941), is undoubtedly associated with the presence of lignin. In caustic soda, on the other hand, swelling occurs in low concentration, with a sudden rise in percentage swelling between 5 and 8 per cent. caustic soda followed by a slow progressive increase up to about 15 per cent. caustic soda. At this point the increase in wall thickness is 129 per cent. and in diameter 57 per cent. and the lumen is occluded. If pressure is applied without disorganizing the wall the corresponding values

are 148 and 71 per cent. This internal swelling is undoubtedly associated with resistance to swelling of the outermost layers at least, and this can hardly be associated with lignin content since the outer layers in the wall appear to have

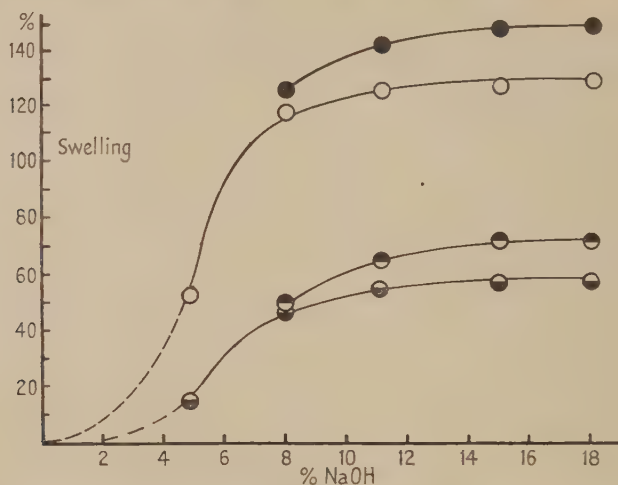


FIG. 4. Swelling of bamboo fibres in caustic soda solutions. —○— ° swelling in wall thickness. —●— Do. after application of pressure. —○— ° swellings in cell diameter. —●— Do. after application of pressure.

less lignin than the inner layers. It seems most likely that the effect is due to a molecular configuration of the outer lamellae different from that in the bulk of the wall and it remains therefore to be decided what this difference, already apparent from optical data, might be. This will be attempted in the following pages.

DISCUSSION

The optical properties of the outer layers of bamboo fibres reported above lend themselves to the implication that the cellulose chains of which they are composed lie along a fairly flat spiral. This possibility can be tested in the following way, using the recorded values of the refractive indices in transverse and longitudinal section. The refractive indices must be referred to an index ellipsoid (Fig. 5) in which the n_y direction lies in the plane of the wall and at some angle θ to the length of the cells. Let $n_y^{\perp}$ be the major refractive index in transverse section and $n_y^{\parallel}$ the major refractive index in sections parallel to cell length. Then, assuming the cellulose to be uniaxial:

$$\frac{(n_y^{\perp})^2 \sin^2 \theta}{n_y^2} + \frac{(n_y^{\parallel})^2 \cos^2 \theta}{n_{\alpha}^2} = 1.$$

Hence

$$(n_y^{\perp})^2 = \frac{n_y^2 n_{\alpha}^2}{n_y^2 - (n_y^2 - n_{\alpha}^2) \sin^2 \theta} \quad (1)$$

Similarly
$$(n_{\gamma}^{\parallel})^2 = \frac{n_{\gamma}^2 n_{\alpha}^2}{n_{\gamma}^2 - (n_{\gamma}^2 - n_{\alpha}^2) \cos^2 \theta} \quad (2)$$

Obtaining $\sin^2 \theta$ from (2) and substituting in (1),

$$n_{\gamma}^2 [(n_{\gamma}^{\parallel})^2 (n_{\gamma}^{\perp})^2 + n_{\alpha}^2 (n_{\gamma}^{\perp})^2 + (n_{\gamma}^{\parallel})^2 n_{\alpha}^2] = (n_{\gamma}^{\perp})^2 n_{\alpha}^2 (n_{\gamma}^{\parallel})^2.$$

Knowing $n_{\gamma}^{\parallel}$, $n_{\gamma}^{\perp}$, n_{α} (see Table IV), n_{γ} may thus be calculated and hence θ determined from (1) or (2). The value of n_{γ} for both species used turns out to be 1.60 and θ as 34° . The value of n_{γ} closely resembles that measured in ramie by Frey (1926) (1.590) and is identical with the figure reported by Hermans (1946). This suggests, therefore,

that the orientation in the surface of bamboo fibres, with respect to the common direction of the cellulose chains, is as perfect as in ramie. Although this value of n_{γ} may be rather high (see below), the agreement does lend some confidence in the value of 34° for the angle. Since the bulk of the wall within is composed of chains running almost longitudinally, the surface of these fibres is materially different from the rest of the wall. So far, therefore, the structure of bamboo fibres resembles in general outline that of conifer tracheides (Bailey and Kerr, 1935; Wardrop and Preston, 1947) and sisal (Preston and Middlebrook, 1950). It should be emphasized that a similar structure has been demonstrated, therefore, in this laboratory, by the application of strictly legitimate physical principles, in three different cell types using three different methods. One major difference should, however, be noted here between the outer layers in bamboo fibres as compared with wood or sisal. In wood the intrinsic birefringence of the cellulose

is low, of the order 0.02, much lower than could be expected from well-oriented cellulose even if the high lignin content is taken into account. The chains of cellulose there must therefore be considerably dispersed. Similarly in sisal, though the outer layer alone yields a good spiral diagram indicative of well-oriented chains, the completed adult fibre yields a diagram corresponding to the inner layer only. During deposition of the inner layer, therefore, the chains of the outer layer must have become more dispersed about their common direction, or something else must have happened, to reduce the intensity of its X-ray photograph. This could be associated with the onset of lignification. In bamboo, on the other hand, optical evidence suggests that the chains in the outer layer remain well oriented, and if this were indeed the

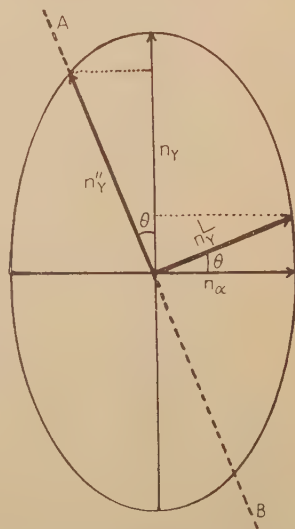


FIG. 5. The projection of the index ellipsoid on the surface of the cell wall. AB parallel to fibre length.

case it could be associated with the apparently low lignin content here. On the other hand, however, the X-ray diagrams of adult fibres do not exhibit any diffraction arcs which might arise from outer layers only. Certainly the 002, 101, and 10 $\bar{1}$ lateral arcs are often spread in a complete circle, but there is no increase in intensity along the circle at regions ($\pm$ about 30° from the equator) where the reflections from the outer spiral might be expected to occur. Weakness of an X-ray diagram is not in itself, of course, inconsistent with high birefringence, for the latter property of a cellulose fibre could reach a high level without the regular spacing of the constituent chains in a space lattice, i.e. with a low X-ray crystalline non-crystalline ratio. Nevertheless, such a condition would be highly unusual and it seems possible that some angular dispersion occurs in the outer layer, in which case the calculated value of n_γ , and therefore of θ , is rather too high. This is to be remembered in interpreting these figures. Taking, however, the value $n_\gamma = 1.60$ at its face value, the spiral angle θ can be calculated for each of the fibre lengths given in Table III; they can be expressed as a linear relation between length and $\cot \theta$. This relation is in the form:

$$L = 5.980 (\cot \theta - 0.95),$$

so that in these outer layers the variation of θ with length is less than with the inner layers. It is also to be noted that, provided the line can be extrapolated to zero length, then no fibre should have an outer spiral flatter than $\theta = 45^\circ$.

Further confirmation of the presence of an outer layer with a fairly flat spiral organization is given by the appearance of isolated cells swollen in 18 per cent. KOH and placed in 63 per cent. H₂SO₄ (Plate I, Fig. 3). The outer layer then shows spiral markings lying at an angle to cell length of about 45°. Although phenomena of this kind are valueless as isolated observations, they are clearly useful as a check on X-ray and optical determinations. As regards the effect of this outer layer on the extent of swelling it is of some interest to note that if we consider the cellulose chains in the outer layer to become flatter as swelling proceeds, then a change from an angle of 34° to one of 90° (transverse orientation) would involve a swelling of 74 per cent. above the diameter in water. This compares favourably with the 71 per cent. found (p. 222) as the maximum observed swelling so long as the wall remained coherent.

It is thus probable that the correlation between $n_\gamma^||$ and cell length, noted above, is to be interpreted in terms of a relation between the outer spiral in the wall with cell length. This in itself offers no explanation of the observed inverse correlation between $n_\alpha^||$ and cell length. Such a correlation could arise through the operation of any one or more of a number of factors. These will be discussed in a later paper. At the moment we note further that, making the assumption that n_γ is the same for all layers which are bright in transverse section, then the spiral for the inner bright layers (where the longitudinal birefringence cannot be determined) is steeper than in the outer layers. This progressive steepening might be associated with decreasing diameter of the

layers much as described for tracheides (Preston, 1948), but the number of layers is too few to attempt any numerical relation.

There is thus in bamboo fibres a good deal of evidence for a simple relationship between cell length and wall structure. This has been demonstrated using (a) major extinction positions, (b) X-ray diagrams both of whole bamboo and separated fibres, (c) optical properties of the outer layer. Since a similar relationship has been found in wood tracheides, sisal fibres, and indicated by workers in other laboratories for cotton and *Musa*, it seems highly probable that it is of rather general application and reflects some fundamental feature of cell wall architecture.

The structure of bamboo fibres is therefore exceptionally complicated and any attempt to interpret properties of bamboo in bulk, in terms of wall structure, must for the moment be approached with caution. In later papers attempts will be made to determine how far such an interpretation can be taken with one or two specific properties.

It is finally to be suggested that bamboo fibres might form admirable material for the elucidation of the factors underlying the so-called 'crossed fibrillar structure' in plant cell walls, and examination of developing fibres would therefore well repay study.

SUMMARY

Following the recent work on wood tracheides and sisal fibres, a detailed investigation has been made of the fine structure of a wide variety of bamboo fibres. A combination of X-ray analysis, measurement of refractive indices in longitudinal view and of phase differences in transverse section, has presented a complete picture of cellulose chain orientation. The wall of bamboo fibres is many layered. An outermost, thin layer is composed of cellulose chains making an angle of 35° to cell length and this is followed by other, inner layers where the angle steadily decreases from outer to inner layers, first to about 20° and then to about 10° . These layers are separated by thicker layers, dark in transverse section between crossed nicols, in which the angle is seldom more than $5-6^\circ$. These are *average* figures. The results show most clearly that each angle, θ , varies with the fibre length, L , in such a way that the longer cells have steeper spirals in harmony with a relation of the type $L = A + B \cot \theta$ already proposed for wood tracheides.

LITERATURE CITED

- ASTBURY, W. T., PRESTON, R. D., and RANGANATHAN, V. (1950). (To be published.)
 BAILEY, I. W., and KERR, T. (1935). *J. Arnold Arbor.* **16**, 273.
 — and VESTAL, M. B. (1937). *Ibid.* **18**, 185, 196.
 COPPICK, S., and FOWLER, W. T. (1939). *Paper Trade J.* **109**, 135.
 FREY-WYSSLING, A. (1926). *Jahrb. wiss. Bot.* **65**, 195.
 HERMANS, P. H. (1946). *Contributions to the physics of cellulose fibres*. Elsevier.
 KUNDU, B. C., and PRESTON, R. D. (1940). *Proc. Roy. Soc., B*, **128**, 214.

- PRESTON, R. D. (1934). *Phil. Trans. Roy. Soc., B*, **224**, 131.
 — (1939). *Proc. Leeds Phil. Soc.* **3**, 546.
 — (1941). *Proc. Roy. Soc., B*, **130**, 103.
 — (1946). *Ibid.* **133**, 327.
 — (1947). *Ibid.* **134**, 202.
 — (1948). *Biochem. et Biophys. Acta.* **2**, 370.
 — and MIDDLEBROOK, MAVIS. (1949). *J. Text. Inst.* (in press).
 REIMERS, H. (1922). *Mitt. deutschen Forsch. Inst. f. Textilstoffe, Karlsruhe*.
 SISSON, W. A., and CLARK, G. L. (1933). *Industr. Engng. Chem. An. Ed.* **5**, 296.
 STEINBRINCK, H. (1927). *Naturwiss.* **15**, 978.
 WARDROP, A. B., and PRESTON, R. D. (1947). *Nature, Lond.* **160**, 911.
 — — (1948). (To be published.)

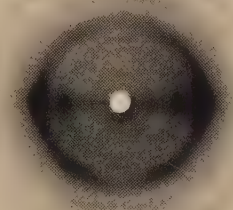
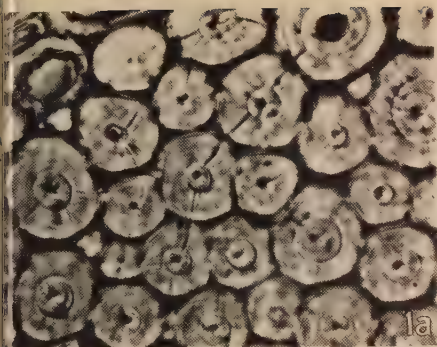
DESCRIPTION OF PLATE I

FIG. 1 (a). Transverse section, 5μ thick, of *Dendrocalamus strictus* as seen under the ordinary microscope.

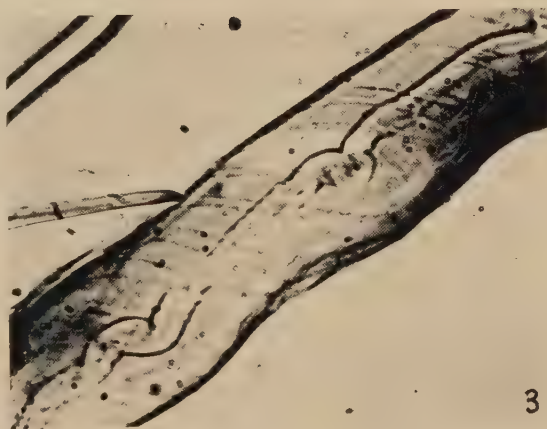
FIG. 1 (b). A similar section as seen under the polarizing microscope between crossed nicols. Note the alternation of bright and dark layers.

FIG. 2. X-ray diagram of untreated material of *D. strictus*. X-ray beam CuK_α from Shearer type tube collimated by a slit 0.5 mm. diameter. Beam perpendicular to fibre length, fibre length parallel to long edge of page. Specimen-film distance, 3 cm.

FIG. 3. Isolated cell of *D. strictus* treated with 18% KOH followed by 63% H_2SO_4 . Notice the spiral markings lying on the outside of the (swollen) cell.



2



3

17

B.

Is:

expe

Palat

conc

The

so re

In th

desce

they

furl

of F

men

type

secr

I.

A

betw

aper

inte

rap

mon

dec

evie

han

Ma

F

ver

Jour

Studies in Stomatal Behaviour

V. THE ROLE OF CARBON DIOXIDE IN THE LIGHT RESPONSE OF STOMATA

PART II. *Preliminary Experiments on the Interrelations of Light Intensity, Carbon Dioxide Concentration, and Rate of Air Flow in Controlling the Move- ment of Wheat Stomata*

O. V. S. HEATH AND F. L. MILTHORPE¹

*Research Institute of Plant Physiology, Imperial College of Science and Technology,
London, S.W. 7*

IN the previous paper of this series (Heath, 1950) is given an account of the experiments which led to the qualitative discovery that stomata, both of *Pelargonium* and wheat, respond markedly to changes of carbon dioxide concentration in the external air between the normal 0.03 per cent. and zero. The changes of stomatal aperture induced were so extreme and the response so relatively rapid that a more quantitative investigation was clearly indicated. In the present paper the preliminary experiments of such an investigation are described. Their publication at this early stage seems desirable both because they embody an important extension of the original finding and because further collaboration between the authors is rendered impossible by the return of F. L. M. to Australia. The wheat leaf was used throughout these experiments as it possesses a number of advantages over that of *Pelargonium* for this type of investigation. These advantages are briefly mentioned in the next section and it is hoped to consider them in detail elsewhere.

EXPERIMENTAL

I. *The effects of carbon dioxide concentration and rate of flow of dry air at different light intensities*

A series of experiments was designed to analyse quantitatively the relation between stomatal resistance to viscous flow, used as a measure of stomatal aperture, and carbon dioxide concentration of the air, at three different light intensities. It had been found that *Pelargonium* stomata responded more rapidly to changes of carbon dioxide concentration and came to an equilibrium more quickly in dry than in moist air (Heath, 1950). For this reason it was decided to use dry air in the present experiments, although there was some evidence that wheat stomata opened less in atmospheres of low than of high humidity.

Materials and methods

Potted plants of wheat (vars. 'Charter' and 'Bencubbin'), 4-6 weeks old, were used in this series and a fully expanded leaf, the fourth or fifth to develop,

¹ This work was carried out while F. L. M. held a Farrer Memorial Scholarship.

was selected for each experiment. A rectangular area of 2.56 sq. cm. (6.4×0.4 cm.) on one side of the midrib and about half-way along the leaf was enclosed between two chambers which are shown diagrammatically in Fig. 1, A and B. The bases of the two leaf chambers were formed of rectangular

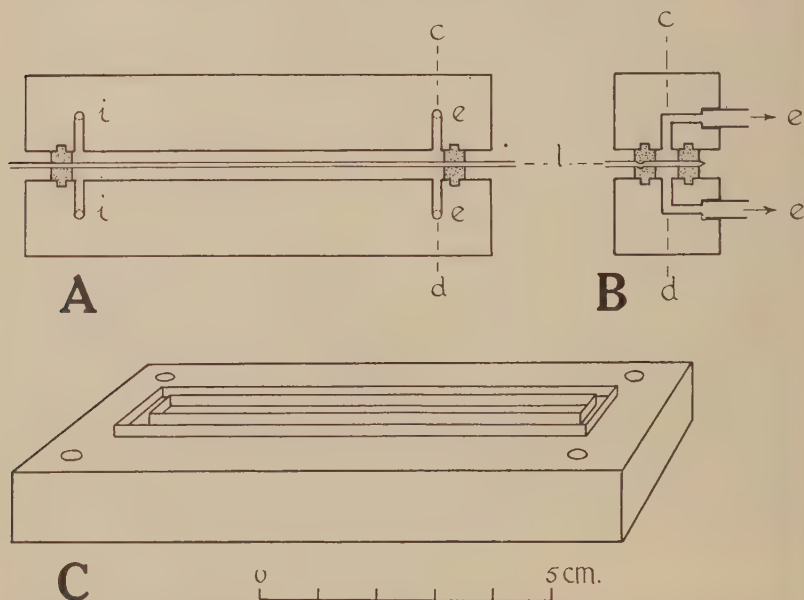


FIG. 1. A. Leaf chambers shown in longitudinal median section. B. The same in transverse section at the level *cd* of the exit tubes *e*. *i*, inlet tubes; *e*, exit tubes; *l*, leaf. Gelatine washers stippled. C. Cutter used in preparing gelatine washers. For further explanation, see text.

blocks of perspex, each $8.0 \times 1.8 \times 1.3$ cm. and suitably grooved to retain a gelatine washer. Inlet and outlet tubes (*i* and *e*) were provided in each block. Exact registration of the two blocks above and below the leaf was ensured by four bolts. The gelatine washers which formed the walls of the chambers were made as follows: molten gelatine¹ was poured to a depth of 2 mm. over the grooved surface and allowed to set thoroughly—preferably overnight. A special cutter (Fig. 1, c) made from pieces of razor blade set in plaster of Paris was then used to remove all the gelatine except a wall 0.35 cm. wide surrounding a rectangular area 6.4×0.4 cm. Exact registration of the upper and lower chambers was achieved by the cutter fitting over the bolts at the four corners of the perspex block. When an experiment was to be set up, the gelatine walls were coated with a mixture of beeswax and stopcock grease ('Apiezon M'), the leaf was then laid upon the lower chamber, the upper chamber placed in position, and the four knurled nuts gently screwed down until freedom from leaks was secured.

¹ 35% gelatine dissolved in a mixture of $\frac{2}{3}$ glycerine and $\frac{1}{3}$ water by weight.

Throughout an experiment, except during actual porometer readings, both chambers were swept with dry air of predetermined carbon dioxide content. The use of balanced push and pull maintained the pressure within the chambers atmospheric at all rates of flow, as indicated by manometers m_2 and m_3 (Fig. 2), thus preventing any tendency either to force air out through the leaf via the stomata or to draw external air through the leaf into the chambers. No 'guard ring' to reduce lateral diffusion of carbon dioxide was provided in these experiments (cf. Heath, 1939), but the concentration difference between the chambers and external air was in all cases small (between 0.00 and 0.03 per cent.). The control of air supply was similar to that described in detail elsewhere (Gregory *et al.*, 1950): a cylinder of compressed air of determined carbon dioxide content (approx. 0.03 per cent.) provided two streams of air at rates of flow controlled by needle valves and measured by flow-meters. One air stream was scrubbed free of carbon dioxide; both were dried and then mixed. By varying the two rates of flow any desired total flow through the chambers with any carbon dioxide content from *c.* 0.03 per cent. to zero could be obtained. Owing to the use of upper and lower chambers in the present experiments, however, the air supplies differed from those described in the paper cited in certain details. These resembled the methods previously employed by one of us (Heath, 1939), *viz.* the mixed air supply was again divided, this time into two equal streams, to sweep the upper and lower chambers respectively by means of two pairs of balanced capillary resistances. Because of the capillary resistances r (Fig. 2) the air was delivered by the flow-meters at a pressure higher than that in the chambers (c). This pressure was indicated by a manometer m_1 and the rates of flow of the carbon dioxide-free and ordinary air streams before mixing were adjusted to provide the required rate of flow through the chambers at atmospheric pressure. Furthermore, taps were so arranged that when a porometer reading was to be taken the exit tubes of both chambers were closed, the lower chamber was shut off from the air supply and used as a porometer cup, while the air of controlled carbon dioxide content escaped to the atmosphere through the three-way tap on the inlet tube to the upper chamber. This ensured that air drawn into the upper chamber and through the leaf by the porometer was of the same carbon dioxide content as that passing over the leaf during the rest of the experiment: contamination by laboratory air was thus avoided. The appropriate positions of the taps are indicated in Fig. 2.

Stomatal resistance to viscous flow of air was estimated at intervals of 20 minutes by means of the resistance porometer (Gregory and Pearse, 1934; Heath, 1941). Both manometers were read with horizontal microscopes and were filled with a light hydrocarbon ('Dekalin') which gave a rapid response and was free from sticking; a gasometer type of aspirator was used in order to obtain steady manometer readings (Heath and Williams, 1948), but since it was used to give a *reduced* pressure the vapour from the Dekalin could not reach the leaf.

In the wheat leaf the ratio, α , of the stomatal number on the upper surface

to that on the lower, is greater than unity; this being so, nearly all the air drawn into the cup by the porometer must pass straight through the leaf from top to bottom and relatively little laterally through the mesophyll from the stomata outside the cup area (Heath, 1941; Penman, 1942). It follows that

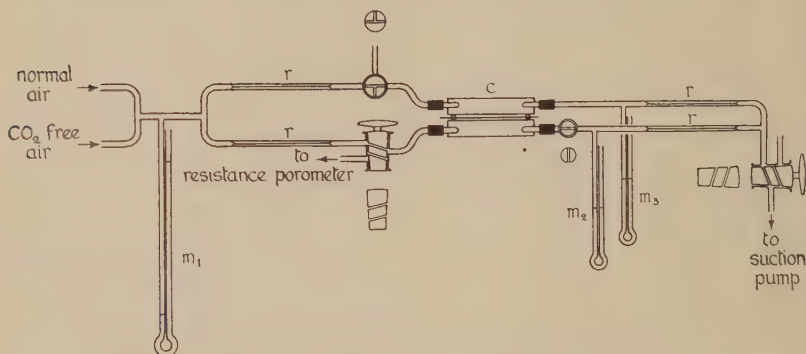


FIG. 2. System of air flow through the leaf chambers. Insets show position of taps during a porometer reading. For further explanation, see text.

neither the stomatal apertures on the rest of the leaf nor the mesophyll resistance can have very much effect upon the total resistance observed by means of the porometer. In the present experiments almost identical conditions were maintained in the upper and lower chambers between readings, and the errors due to differences of stomatal aperture within and outside the porometer cup, stressed in the previous paper (Heath, 1950), are unlikely to have been important here.

After each of the present experiments the portion of leaf within the chambers was carefully cut out and preserved in alcohol, the number of stomata on each surface being subsequently counted under the microscope. In this way the mean value of α was found to be 1.32. From the porometer data values of $\log_e$ (resistance per 10,000 stomata) were calculated, i.e. logarithms of the stomatal resistance to viscous flow for a total of 10,000 stomata within the cup area (including both surfaces) and a value of α of 1.32. This resistance may be approximately estimated as

$$R_{10,000} = R_{\text{observed}} \left(\frac{n_u n_l}{n_u + n_l} \times \frac{10,000}{5690 \times 4310} \right),$$

where n_u and n_l are the stomatal numbers on the upper and lower surfaces respectively and 5690 and 4310 are the numbers of stomata, on the upper and lower surfaces respectively, to give a total of 10,000 and a ratio α of 1.32. The use of this expression assumes:

1. That lateral flow through the mesophyll from stomata external to the cup area is negligible.
2. That the rate of air flow varies directly as the pressure difference.

3. That the conductances of the upper and lower epidermes are in the ratio of the numbers of stomata, i.e. that mean stomatal apertures are at all times the same on both surfaces and that interference between stream lines flowing through neighbouring stomata is negligible or similar for both surfaces.
4. That the intercellular space resistance normal to the leaf surface is negligible.

All experiments were carried out in a constant temperature room maintained at 25° C. The details of the source of light are given in Gregory *et al.* (1950); intensities of approximately 275, 625, and 975 foot candles, as measured with a photo-electric meter, were used in the present experiments.

It was at first intended to investigate the effects of different carbon dioxide concentrations at the various light intensities using a single rate of flow. For this purpose the attempt was made to find a rate of flow so high that with the highest light intensity and carbon dioxide content (975 f.c. and 0.036 per cent.) any further increase in rate of flow caused no further appreciable closure of the stomata. It was found, however, that under these conditions substantial stomatal closure occurred with increased rate of flow up to 16 l./hr., the highest rate tested. At this rate of flow the carbon dioxide supply was at the rate of 441 mg. per sq. dm. of leaf surface per hour and a closing effect of high wind speed apart from the carbon dioxide effect was strongly suggested. It was therefore decided to include rate of air flow as a further factor.

Four experiments are included in the series under discussion: one at a light intensity of 975 f.c., two at 625 f.c., and one at 275 f.c. A synopsis of these is presented in Table I. In each experiment four different carbon dioxide concentrations (c. 0.00, 0.01, 0.02, and 0.03 per cent.) were given in a random order as four successive treatments on a single day at one rate of flow. This was repeated at three rates of flow (2, 5, and 12.5 l./hr.) which were used on successive days, again in a random order. There were thus twelve 'treatments' in each experiment. With 'high' (975 f.c.) and 'medium' (625 f.c.) light intensities it was possible to use the same leaf for three consecutive days and thus carry out the whole experiment on a single leaf. With 'low' light (275 f.c.) it was found, however, that even on the second day the stomata did not open as widely as on the first day with the same treatment, and by the third day the leaf was definitely yellowing. In this experiment (No. 17), therefore, two leaves were used: leaf *A* was given the four carbon dioxide concentrations at 5 l./hr. on the first day and at 2 l./hr. on the second; leaf *B* at 12.5 l./hr. on the first day and 2 l./hr. on the second. Thus any difference between the leaves is confounded with the two rates of flow 12.5 l./hr. and 5 l./hr., while the results for the 2 l./hr. rate of flow are not comparable with the others and must be considered separately.

The carbon dioxide concentration of the air leaving the leaf chambers was not determined and hence the mean concentrations to which the leaf was exposed could not be estimated. The $\log_e$ stomatal resistance was therefore

plotted against carbon dioxide concentration of the air entering the chambers. A different cylinder of air was used for the low light experiment (No. 17) and this gave slightly higher concentrations of carbon dioxide as shown in Table I. These differences have been ignored in plotting the diagrams

TABLE I
Synopsis of experiments. Series I

Expt. No. and dates	Light inten- sity, f.c.	Variety	Order of treatments					
			1st day		2nd day		3rd day	
			Air flow l./hr.	CO ₂ %	Air flow l./hr.	CO ₂ %	Air flow l./hr.	CO ₂ %
14 (3-5.6.48)	975	Charter	5	0.020	2	0.010	12.5	0.030
				0.000		0.000		0.000
				0.010		0.030		0.010
				0.030		0.020		0.020
16 (21-23.6.48)	625	Charter	5	0.020	12.5	0.000	2	0.030
				0.010		0.010		0.010
				0.000		0.020		0.020
				0.030		0.030		0.000
11 (13-15.4.48)	625	Bencubbin	2	0.000	12.5	0.020	5	0.030
				0.010		0.030		0.010
				0.020		0.010		0.020
				0.030		0.000		0.000
17A (29-30.6.48)	275	Charter	5	0.033	2	0.000	—	—
				0.011		0.011		
				0.022		0.033		
				0.000		0.022		
17B (1-2.7.48)	275	Charter	12.5	0.000	2	0.011	—	—
				0.022		0.022		
				0.011		0.033		
				0.033		0.000		

(Figs. 7 and 8). It was originally intended to carry out the twelve 'treatments' at each light intensity in duplicate, using the two varieties 'Charter' and 'Bencubbin', as was done for the medium light level. The series was, however, discontinued before completion for two reasons: first, that it was impossible to carry out the twelve treatments at the low light on a single leaf, as stated above, and secondly that a separate investigation of the 'wind-speed effect' was clearly a prior necessity.

The experimental technique was to follow stomatal movement with the porometer at intervals of 20 minutes until, under a given set of conditions, an equilibrium value was reached and maintained for 40 minutes (three successive readings); this was taken as giving the stomatal resistance for that particular treatment. The carbon dioxide concentration was then changed and a further treatment value obtained. The leaf was generally set up overnight before the first day's experimentation; it was kept in darkness and still air during both this and each subsequent night until the end of the experiment.

It has been stated above that an equilibrium value, when the porometer reading had been steady for 40 minutes, was taken as the resistance corresponding to each set of experimental conditions. In practice, however, some difficulty was experienced in deciding upon these values as the porometer readings seldom achieved a completely steady level, apparently for two reasons. In the first place there was sometimes a tendency for 'overshooting' to occur when a change of conditions caused stomatal opening or closure, the stomata

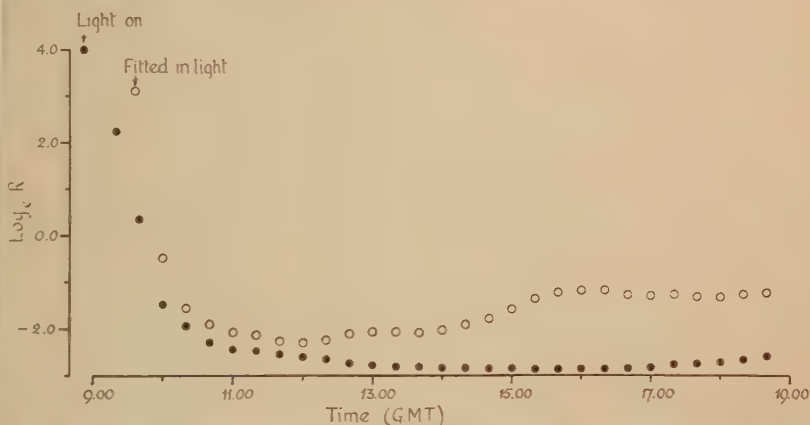


FIG. 3. Experiment 18. Trend of $\log_e$ stomatal resistance throughout the first (○) and third (●) days after fixing porometer cup. Light, 625 f.c.; air flow, 5 l. hr.; CO_2 , 0.01% on first day and 0.00% on third day.

subsequently recovering to give a less extreme value of resistance. This has also been found in unpublished work with onion leaves. In the second place the results often gave evidence of a diurnal effect with a marked closing tendency in the afternoon. Some time-curves are presented in Figs. 3 and 4 to illustrate these effects. In Fig. 3, where a single treatment was given throughout the day, marked diurnal closure occurred during the latter part of the first but not of the third day. In Fig. 4 some time-curves from Expts. 14 and 17B, which provided some of the data for this series, illustrate the difficulty of deciding on the 'equilibrium values'. The portions actually selected for calculating the mean resistance at each 'equilibrium' are indicated by the brackets thus ——. It is clear that the exact values of these 'equilibria' are in some doubt, but since the treatments were given in a random order, *systematic* errors due to a diurnal rhythm should have been eliminated. Errors due to 'overshooting' probably occurred in some cases, but are unlikely to have been large compared with the treatment effects.

Results

Expt. 14. 975 f.c. var. 'Charter'. The results of this experiment are presented in Fig. 5 where $\log_e$ stomatal resistance is plotted against rate of air flow and carbon dioxide concentration. The results for a rate of air flow of

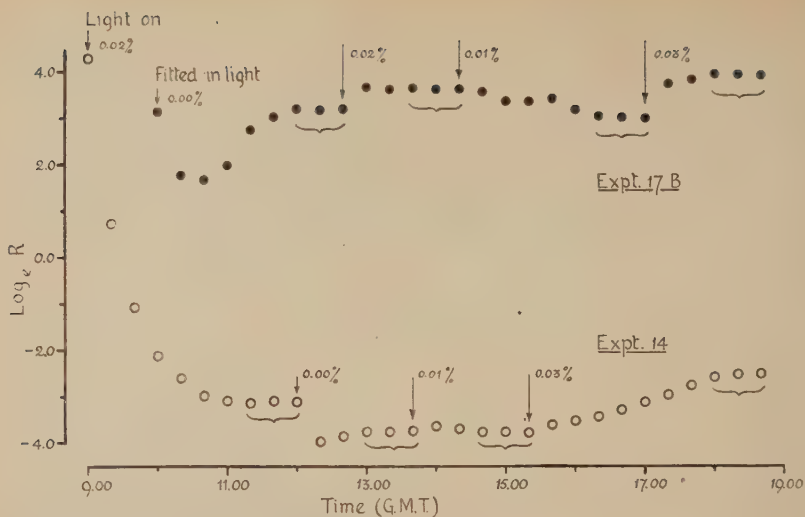


FIG. 4. Time-curves for $\log_e$ stomatal resistance from Experiment 14 (975 f.c., 5 l./hr.) and Experiment 17B (275 f.c., 12.5 l./hr.). Changes of CO₂ concentration indicated by arrows.

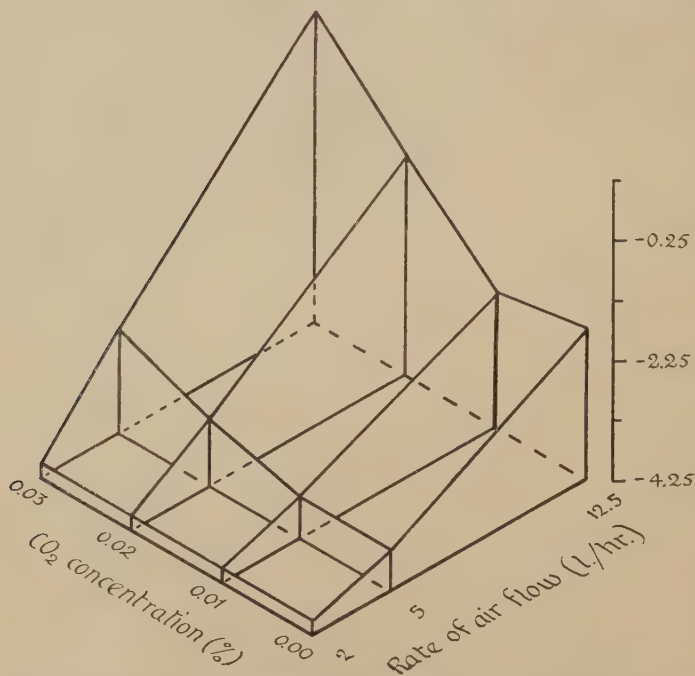


FIG. 5. Experiment 14 (var. 'Charter'). Light intensity 975 f.c. Isometric projection of 3-dimensional diagram showing relation of $\log_e$ stomatal resistance to rate of air flow and CO₂ concentration.

5 l./hr. are also incorporated in Fig. 7, and those for 12.5 l./hr. in Fig. 8, where $\log_e$ stomatal resistance is similarly plotted against *light intensity* and carbon dioxide concentration.

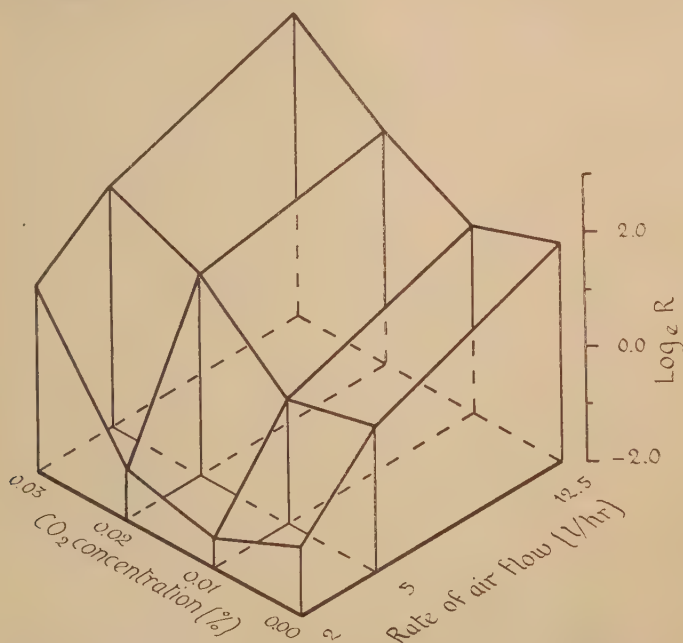


FIG. 6. Experiments 11 (var. 'Bencubbin') and 16 (var. 'Charter'). Light intensity 625 f.c. Relation of mean $\log_e$ stomatal resistance from the two experiments to rate of air flow and CO_2 concentration.

Expt. 11. 625 f.c. var. 'Bencubbin'; *Expt. 16.* 625 f.c. var. 'Charter'. Analysis of variance on the results of this pair of experiments showed no significance for the mean difference of $\log_e$ (resistance per 10,000 stomata) between the two leaves ('Charter', 1.10; 'Bencubbin', 0.70; $P > 0.30$). Mean results for the two experiments are given in Fig. 6 which is in the same form as Fig. 5, and also in Table II, where the differences required for significance at the $P = 0.05$ level are shown in brackets.

TABLE II

Experiments 11 (var. 'Bencubbin') and 16 (var. 'Charter'). Light intensity 625 f.c. Mean $\log_e$ (resistance per 10,000 stomata) for α 1.32, from both experiments. (In brackets: difference required for $P = 0.05$)

Rate of air flow l./hr.	CO ₂ concentration				Mean (0.64)
	0.000	0.010	0.020	0.030	
2	-0.82	-1.50	-1.16	+1.20	-0.57
5	+0.53	+0.16	+1.53	+2.18	+1.10
12.5	+1.81	+1.24	+2.04	+3.23	+2.08
Mean (0.76)	+0.51	-0.03	+0.80	+2.20	

Highly significant average effects were found both for rate of air flow ($P = 0.01$) and carbon dioxide concentration ($P < 0.001$). It will be seen that each increase in rate of flow caused, on the average, a significant increase in log stomatal resistance; high carbon dioxide caused significant stomatal closure, but the apparent closure with reduction of carbon dioxide from

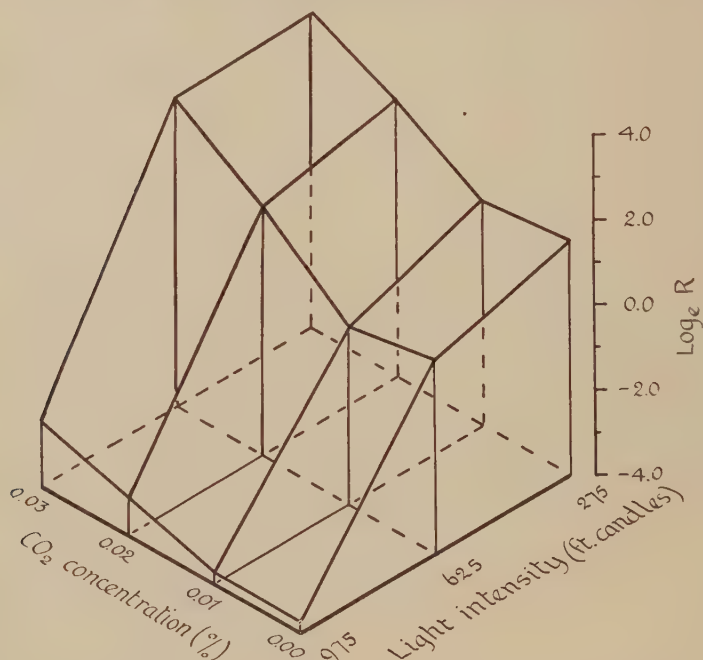


FIG. 7. Experiments 14, 16, and 17*A* (var. 'Charter'). 5 l./hr. air flow. Relation of $\log_e$ stomatal resistance to light intensity and CO_2 concentration.

0.01 per cent. to zero failed to reach significance. The interaction of rate and concentration did not approach a significant level and hence only average effects should be discussed.

The above data (var. 'Charter' only) for the 5 l./hr. rate of air flow are also included in Fig. 7 and for 12.5 l./hr. in Fig. 8.

Expts. 17 A and B. 275 f.c. var. 'Charter'. As already mentioned (p. 231 and Table I) two leaves were used. The data obtained on the first days, with rates of flow of 5 l./hr. in 17*A* and 12.5 l./hr. in 17*B*, are incorporated in Figs. 7 and 8 respectively. A comparison of the two curves gives no indication of any interaction between rate of air flow and carbon dioxide concentration. The log stomatal resistance obtained in the second day's experimentation, with a rate of air flow of 2 l./hr., was at each carbon dioxide content higher than the corresponding value found at 5 l./hr. on the first day in Expt. 17*A*. These results at 2 l./hr. are clearly not comparable with the others, as the leaves

were apparently already suffering from the low light intensity, but they provide some corroborative evidence for the carbon dioxide effect and are presented in Table III.

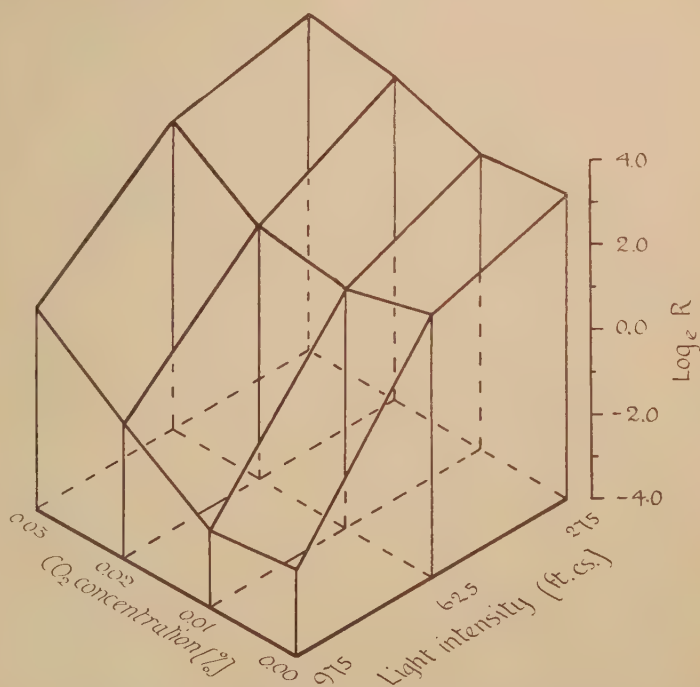


FIG. 8. Experiments 14, 16, and 17B (var. 'Charter'). 12.5 l./hr. air flow. Relation of $\log_e$ stomatal resistance to light intensity and CO_2 concentration.

TABLE III

Experiments 17 A and B (var. 'Charter'). Light intensity 275 f.c. Rate of air flow 2 l./hr. Second day's results. $\log_e$ (resistance per 10,000 stomata) for $\propto 1.32$. (In brackets: difference required for $P = 0.05$)

Expt.	CO_2 concentration				Mean (0.86)
	0.000	0.011	0.022	0.033	
17A	1.65	1.40	3.83	3.87	2.69
17B	2.35	2.10	3.57	3.67	2.92
Mean (1.21)	2.00	1.75	3.70	3.77	2.81

A significant average effect of carbon dioxide concentration was shown by these two replicates ($P < 0.05$).

It must be noted that, for convenience, very different base lines have been used in Fig. 6 as against Figs. 5, 7, 8, and also that different vertical scales have been used in Figs. 5, 6, and 7, 8. These differences should be allowed for in comparing one diagram with another.

Figs. 5 and 6, showing the results of Expt. 14 and Expts. 11 and 16 respectively, represent entirely independent evidence and this also applies to the low light-curves in Figs. 7 and 8, and to the data in Table III for 2 l. hr. air flow at low light. Some of these data are again presented in Figs. 7 and 8 to illustrate the light intensity effects, and although these two diagrams are independent of each other, with the exception of the low light curves they must not be taken as providing *additional* evidence of the carbon dioxide effects seen in Figs. 5 and 6 and Table III. An overall analysis of variance on the data has not been attempted in view of the incompleteness of the series (see p. 232); it is clear, however, from an inspection of the independent data as enumerated above that each of the three factors investigated, viz. carbon dioxide concentration, rate of air flow, and light intensity, has had a very large and significant average effect on stomatal aperture as measured in terms of log resistance.

The most novel and important information gained is that whereas from the normal 0.03 per cent. down to 0.01 per cent., reduction in carbon dioxide concentration causes considerable stomatal opening, from 0.01 per cent. to zero concentration there is no evidence of any further opening. On the contrary, the data rather suggest that some closure occurs over the latter range, though such closure fails to reach statistical significance where tested (Tables II and III) and is not seen at the high light intensity except at the highest rate of flow (Fig. 5).

The large and obviously significant average effect of light intensity seen in Figs. 7 and 8 is perhaps not at first sight surprising. It is of much greater magnitude than the effect of external carbon dioxide and the curves are predominantly parallel, showing that there is little interaction between the two factors (in terms of $\log_e R$). In each of these two diagrams, however, there is some indication of an interaction which, although probably not statistically significant, is worth considering as illustrating certain possible effects. At 12.5 l. hr. air flow (Fig. 8) the apparent interaction is such that the carbon dioxide effect increases appreciably with light intensity. It has already been stressed (p. 231) that the carbon dioxide concentrations plotted do not represent the mean values within the chambers but only the concentration in the air on entry. Owing to the very efficient removal of carbon dioxide from air passing over leaves in such very shallow chambers (Heath, 1949), the differences of mean concentration to which the leaf was exposed were certainly much smaller than those plotted. This would especially be the case with the lower rates of flow at high light intensity, and an extreme example is seen in Fig. 5, where with 2 l./hr. air flow at 975 f.c. light intensity the carbon dioxide effect has entirely disappeared. With 12.5 l./hr. air flow (Fig. 8) the apparent interaction, if a real effect, suggests: (a) that even at the highest light intensity, with wide open stomata and presumably high assimilation rates, sufficient carbon dioxide was supplied by the 0.03 per cent. concentration to influence the tension in the substomatal cavities to a greater extent than at the lower intensities, and therefore (b) that at the latter the relatively small carbon

dioxide effects were due, not to excessive depletion of the supply, but to high stomatal diffusive resistance, resulting in the internal concentration being controlled mainly by photosynthetic activity and much less by external concentration. At 5 l./hr. air flow (Fig. 7) the apparent interaction takes the form of a greater carbon dioxide effect at 625 f.c. than at either the higher or lower light intensity. Here the small effect at 275 f.c., with the stomata relatively closed, might have been due to high diffusive resistance as above, and at 975 f.c. it was probably due to excessive depletion of the supply. It is clear, however, that too much importance must not be attached to relatively small interactions which have not been shown to be statistically significant, especially as the relations of $\log_e R$ either to actual stomatal aperture or to *diffusive* resistance are at present quite uncertain. The most puzzling feature of the data in Figs. 7 and 8 is precisely that there is so *little* interaction, and in particular that light intensity still affects stomatal aperture with an external supply of 0.01 per cent. carbon dioxide (p. 241).

The effect of rate of air flow is also of considerable interest, and especially the fact that increased rate of flow causes stomatal closure at *all* carbon dioxide concentrations. At 975 f.c. light intensity (Fig. 5) there is a marked interaction between rate of flow and carbon dioxide, and the suggestion has already been put forward that this is due to excessive depletion of the carbon dioxide at the lower rates of flow. Here part at least of the rate of flow effect is due to the rate of supply of carbon dioxide. At 625 f.c. (Fig. 6), however, with higher stomatal resistances, no significant interaction is found (Table II). There is thus here no satisfactory evidence that the effect of rate of flow differed with carbon dioxide concentration and therefore no evidence that it was due to rate of carbon dioxide supply. At 275 f.c. (5 and 12.5 l./hr.) there is similarly no indication of an interaction of flow rate and carbon dioxide (see p. 236). At all light intensities, moreover, marked stomatal closure was caused by increasing the rate of flow of *carbon dioxide-free air*. This clearly could not be a carbon dioxide effect, unless the apparent closure found from 0.01 to 0.00 per cent. were real and the air flow effect then due to removal of carbon dioxide from the intercellular spaces. It seemed improbable, however, that this effect would then be of the same magnitude as the air-flow effect at other carbon dioxide concentrations. Taken as a whole, therefore, the evidence strongly suggested some closing effect of increased flow independent of carbon dioxide. Since dry air was used in these experiments, the closure might be due to desiccation and some preliminary data (Heath, 1950) suggested this; on the other hand, some mechanical effect of air movement might be involved. The experiment described in the next section was designed to decide between these alternatives.

II. *The effect of humidity and rate of flow of carbon dioxide-free air (Expt. 19)*

Methods

The same methods were employed as in series I, but with a single light intensity (625 f.c.). In order to eliminate any effect of increased carbon dioxide supply to the stomata with increased rate of flow, only carbon dioxide-free

air was used; this was supplied at rates of 2 l./hr. or 12.5 l./hr. and was either dried with phosphorus pentoxide or humidified by means of a bead tower containing boiled water. There were thus four treatments. The experiment was designed as a 4×4 Latin square, in which the rows represented different leaves and the columns order of treatment. Each leaf was used for two successive days and was given two 'treatments' on each. The periods of the treatments were: (1) from 9.40 a.m. to 3.20 p.m. (opening from darkness), and (2) from 3.20 p.m. to 7.40 p.m. In each case the mean of the last five readings was taken as the stomatal resistance corresponding to that particular treatment. The design of the experiment allowed all four treatments to be carried out on each leaf and each treatment to be given once in each of the four periods. Thus any effect of inherent diurnal rhythm, or change in response with time from setting up, should be eliminated from the treatment means. Actually, however, no significant effect of time was found, nor were the differences between leaves significant.

Results

The mean data from the four replicates are presented in Table IV.

TABLE IV

Experiment 19 (var. 'Charter'). Light intensity 625 f.c. CO₂-free air. Mean log_e(resistance per 10,000 stomata) for $\alpha = 1.32$ from four replicates. (In brackets: difference required for $P = 0.05$)

Rate of air flow l./hr.	Humidity of air		Mean (1.23)
	Dry	Moist	
2	-1.76	-1.67	-1.72
	(1.74)		
12.5	+0.13	-2.73	-1.30
Mean (1.23)	-0.82	-2.20	

Since the interaction of rate of flow and humidity was found significant ($P < 0.05$), the average effects are of little interest. The interaction was such that, with dry air, increasing the rate of flow from 2 to 12.5 l./hr. caused marked and significant stomatal closure, whereas, with moist air, the stomata opened, though not significantly. It seems clear from these results that the closing effect of increased rate of flow found in series I was an effect of desiccation due to the use of air dried with phosphorus pentoxide and not of air movement *per se*.

DISCUSSION

It was found in *Pelargonium* that when the stomata were practically closed the external carbon dioxide concentration ceased to affect their movement, indicating that the concentration in the substomatal cavities rather than at the outer surface of the guard cells was the controlling factor (Heath, 1950). If this also applies to wheat, since an external supply of about 0.01 per cent. carbon dioxide apparently gives maximal stomatal opening at each combination of light intensity and rate of flow tested, it would seem that this must be

the *internal* concentration giving maximal opening. This, if true, is of considerable interest, for assimilation experiments, with air passed *through* the intercellular space systems of brightly illuminated *Pelargonium* and *Begonia* leaves to give various rates of carbon dioxide supply, showed that the air emerging had an almost constant carbon dioxide content of approximately 0.01 per cent. This value was thus indicated as the *minimum* concentration at the surface of the assimilating mesophyll cells (Heath, 1939, 1949). Gabrielsen's (1948) experiments, in which illuminated elder leaves enclosed either in ordinary air or air of 0.0024 per cent. carbon dioxide content brought the concentration to a constant value of 0.009 per cent., also indicate that this is the minimum internal carbon dioxide content. If a similar value holds for wheat leaves, it would seem that the wheat stomata respond to carbon dioxide only down to the minimum concentration that normally occurs in the sub-stomatal cavities. Any response below this level would of course be of no biological advantage to the plant, and even if the apparent slight closure over the range 0.01 per cent. to zero suggested by the data is real, it would appear to have no functional significance.

The large light-intensity effects upon the stomata shown in Figs. 7 and 8, and also the very small amount of interaction between the factors light intensity and external carbon dioxide concentration supplied, have already been stressed (pp. 238-9). If a concentration of 0.01 per cent. carbon dioxide in the sub-stomatal cavities in fact results in maximal stomatal opening, it would be expected that light intensity over a wide range would be without effect at this *external* concentration. That the light effect is as large with 0.01 per cent. carbon dioxide supply as at any other concentration suggests that there is some other important effect of light on the stomata, independent of that acting indirectly through photosynthesis by the mesophyll cells and the carbon dioxide concentration in the substomatal cavities.¹ Such an effect might perhaps operate through photosynthesis in the guard cells themselves, but if so it would seem that they must have an unusually high respiration rate relative to their assimilation rates as otherwise stomatal aperture would be maximal at all but the lowest light intensities. An alternative explanation has been implied earlier (p. 238) in the suggestion that with stomata relatively closed at the lower light intensities the internal carbon dioxide concentration was controlled mainly by photosynthetic activity. It may be that the internal concentration is only reduced to 0.01 per cent. at high light intensities, such as 2,450 f.c. (Heath, 1949) or 1,100 f.c. (Gabrielsen, 1948), and with 625 or 275 f.c. the minimum values may be very much higher. In this case, however, one would expect to find *some* evidence of stomatal opening on reducing the external concentration from 0.01 per cent. to zero at these lower light intensities, but the converse tendency is seen.

It is clear that to investigate these problems further the first essential is to

¹ In this connexion must be mentioned the failure to obtain a light response in stomata on detached strips of epidermis, but this would not appear to be solely due to the absence of the photosynthetic activity of the mesophyll (Heath, 1949*a*, pp. 190 and 205).

control the carbon dioxide concentration in the substomatal cavities to known values and for this purpose it is intended to carry out 'through' experiments (Heath, 1939, 1949) at different light intensities with wheat leaves. Meanwhile, the present preliminary results do suggest the inadequacy of the hypothesis that stomatal control is *entirely* dependent upon the intercellular space atmosphere as affected by respiration and photosynthesis in the mesophyll cells, which was mentioned in the previous paper of this series and has had a number of adherents (this volume, p. 55).

In view of the importance of the drying effect of the air flow shown by the experiment described in Section II it is most desirable to check the results obtained in Section I with this factor eliminated, and the proposed 'through' experiments would in any case have to be carried out with moist air. A large factorial experiment, with humidity of the air flow as one of the factors, has been carried out by methods described in detail elsewhere (Gregory *et al.*, 1950), mainly to investigate effects upon transpiration. The porometer data show large variation between leaves and detailed analysis has not yet been completed. These results will be published separately if they throw new light on the present problems.

It is intriguing to speculate upon the reasons for the very severe 'closing effect' of dry moving air in wheat and its apparent absence in *Pelargonium* (Heath, 1950). It is possible that, in wheat, the lowered leaf water content resulting from increased transpiration rate may be the cause. A system in which stomatal closure may be expected on reduction of leaf turgor is provided in the wheat leaf by the parallel venation and the thin-walled epidermal cells, the so-called 'motor cells' (Percival, 1921), situated between the parallel rows of stomata. In the *Pelargonium* leaf the main veins form a relatively rigid support which might prevent shrinkage in area of the intervening tissues, and the stomata are not oriented in one direction as in wheat. The apparent absence in the present experiments of 'preliminary opening', such as is found with rapid wilting (Darwin and Pertz, 1911; Laidlaw and Knight, 1916; Gregory *et al.*, in preparation), might have been due to the water loss being sufficiently gradual for the guard cells to lose water to the epidermal cells almost as quickly as the latter lost it to the transpiring mesophyll; alternatively the opening may have been too transient to be detected by the porometer readings made at 20-minute intervals. The closing effect of dry air may, however, be due not to turgor changes of the leaf as a whole but to direct loss of water vapour from the guard cells into the air stream. If this is the correct explanation, the absence of the effect in *Pelargonium* may be due to lower water permeability of the guard cell walls, and the thin walls of the dilated ends of the wheat guard cells are suggestive in this connexion. The suggestion made in the previous paper that in the wheat leaf 'the protective function of the carbon dioxide response is reinforced by a direct response to low atmospheric humidity' is supported by the results presented here.

SUMMARY

Experiments are described in which the responses of wheat stomata to carbon dioxide concentration (0.00, 0.01, 0.02, 0.03 per cent.), light intensity (275, 625, 975 f.c.), and rate of air flow (2, 5, 12.5 l./hr.) were studied.

Reduction of carbon dioxide concentration from 0.03 to 0.01 per cent. resulted in marked stomatal opening; further reduction to 0.00 per cent. was accompanied by a slight but not significant closure. These effects were found at all light intensities and rates of flow, except at 975 f.c. with 2 l./hr. air flow, where no effect of carbon dioxide concentration was detected. This last is attributed to excessive depletion of the carbon dioxide supply by assimilation. The apparent lower limit of 0.01 per cent. carbon dioxide causing maximal stomatal opening is discussed in relation to recent assimilation experiments.

Increase of light intensity caused considerable stomatal opening, this effect being as great with air of 0.00 and 0.01 per cent. as with higher concentrations of carbon dioxide. This suggests an effect of light on stomatal movement other than that exerted indirectly through photosynthesis by the mesophyll cells.

Increased rate of flow of dry air caused closure of the stomata; this was shown to be a drying effect and was absent when moist air was used.

LITERATURE CITED

- DARWIN, F., and PERTZ, D. F. M. (1911). On a new method of estimating the aperture of stomata. *Proc. Roy. Soc., B*, **84**, 136-54.
- GABRIELSEN, E. K. (1948). Threshold value of carbon dioxide concentration in photosynthesis of foliage leaves. *Nature*, **161**, 138.
- GREGORY, F. G., MILTHORPE, F. L., PEARSE, H. L., and SPENCER, H. J. (1950). Experimental studies of the factors controlling transpiration. I. Apparatus and technique. *J. Exp. Bot.*, **1**, 1-14.
- and SPENCER, H. J. (in preparation). Experimental studies of the factors controlling transpiration. III. The relation between transpiration rate and stomatal aperture.
- and PEARSE, H. L. (1934). The resistance porometer and its application to the study of stomatal movement. *Proc. Roy. Soc., B*, **114**, 477-93.
- HEATH, O. V. S. (1939). Experimental studies of the relation between carbon assimilation and stomatal movement. I. Apparatus and technique. *Ann. Bot., N.S.*, **3**, 469-95.
- (1941). Experimental studies of the relation between carbon assimilation and stomatal movement. II. The use of the resistance porometer in estimating stomatal aperture and diffusive resistance. Part 1. A critical study of the resistance porometer. *Ibid. N.S.*, **5**, 455-500.
- (1949). Carbon dioxide in the intercellular spaces of leaves during photosynthesis. *Nature*, **164**, 822.
- (1949a). Studies in stomatal behaviour. II. The role of starch in the light response of stomata. Part 1. Review of literature and experiments on the relation between aperture and starch content in the stomata of *Pelargonium zonale*. *New Phytol.*, **47**, 186-211.
- (1950). Studies in stomatal behaviour. V. The role of carbon dioxide in the light response of stomata. Part 1. Investigation of the cause of abnormally wide stomatal opening within porometer cups. *J. Exp. Bot.*, **1**, 29-62.
- and WILLIAMS, W. T. (1948). Studies in stomatal action. Adequacy of the porometer in the investigation of stomatal aperture. *Nature*, **161**, 178.
- LAIDLAW, C. G. P., and KNIGHT, R. C. (1916). A description of a recording porometer and a note on stomatal behaviour during wilting. *Ann. Bot.*, **30**, 47-56.
- PENMAN, H. L. (1942). Theory of porometers used in the study of stomatal movements in leaves. *Proc. Roy. Soc., B*, **130**, 416-34.
- PERCIVAL, J. (1921). *The wheat plant*. London. Duckworth & Co.

A Convenient Volumenometer for Biological Work

P. E. WEATHERLEY

Department of Botany, University of Manchester

METHODS of applying Boyle's Law for measuring volumes of solid objects are described in the standard text-books of physico-chemical methods. Yet volumenometers do not seem to have found much favour in biological research although the problem of measuring the volumes of small irregular objects such as germinating seeds, or samples of roots, is frequently encountered. Volumenometers have the advantage over the more usual displacement methods in that the samples do not have to be immersed in liquid. The apparatus described below, whilst similar in principle to that of Surdo (see *Physico-chemical Methods*, Reilly and Rae, 3rd edition, p. 473), has certain new features and gives a direct reading of the volume of a sample.

Principle

It will be seen from the diagram that a volume of air (V_2) in the pipette P can be compressed into either of the two canisters C_1 and C_2 , by raising the mercury reservoir R so that the mercury level in the pipette rises from p_1 to p_2 . If the initial pressure in the two canisters is the same (atmospheric) and their volumes identical (V_1), a volume V_2 , compressed by the pipette into each in turn, will not result in any pressure difference between the canisters. When, however, this operation is repeated with an object v_1 placed inside the canister C_1 , the final pressure in C_1 will be higher than that in C_2 and a pressure difference will be registered by the manometer M . If the vertical tube H is filled with mercury to the level h_1 , the pressure in C_1 can be reduced by running mercury out through the tap T_5 , since this increases the effective volume of C_1 . The height of the mercury in H can be so adjusted that no pressure difference is registered by the manometer. If this height is h_2 , the volume of the tube between h_1 and h_2 gives a measure of the volume of the object v_1 . This can be shown by a simple application of Boyle's Law.

Let V_1 = volume of canister C_1 including the connecting tubes to p_2 , h_1 , and the manometer tube to the level m (zero pressure difference).

Also V_1 = volume of canister C_2 with connecting tubes to p_2 and to the level m in the manometer.

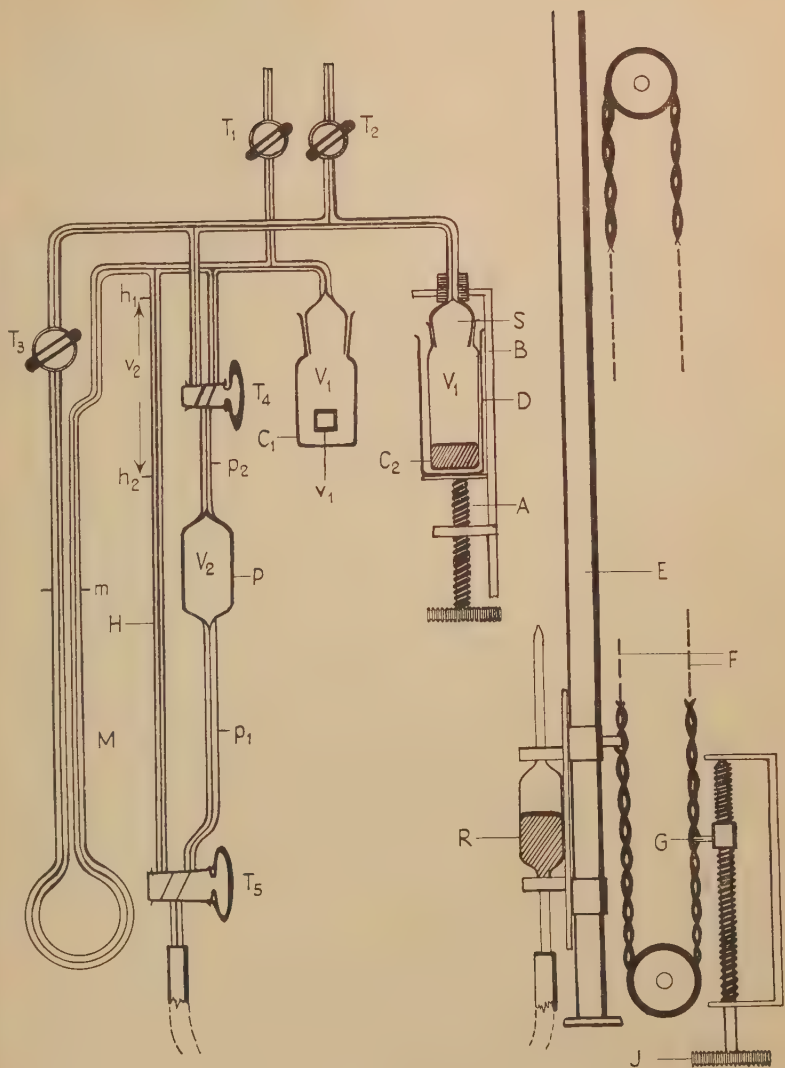
V_2 = volume of pipette between p_1 and p_2 .

v_1 = volume of object to be measured.

v_2 = volume of tube H between h_1 and h_2 .

P_A = atmospheric pressure.

P = final pressure in canisters after equalizing.



For canister C_1 :

Initial pressure = P_A (tap T_1 open).

„ volume = $(V_1 + V_2 - v_1)$.

Final pressure = P .

„ volume = $(V_1 - v_1 + v_2)$,

whence $P_A(V_1 + V_2 - v_1) = P(V_1 - v_1 + v_2)$. (i)

For canister C_2 :

Initial pressure = P_A (tap T_2 open).

„ volume = $(V_1 + V_2)$.

Final pressure = P .

„ volume = V_1 ,

whence $P_A(V_1 + V_2) = PV_1$. (ii)

(ii) divided by (i):
$$\frac{(V_1 + V_2)}{(V_1 + V_2 - v_1)} = \frac{V_1}{(V_1 - v_1 + v_2)},$$

which simplifies to:
$$v_1 = \frac{v_2(V_1 + V_2)}{V_2}.$$

It will be seen that for an apparatus of given dimensions the measured volume v_2 is a definite ratio of the unknown volume v_1 . The apparatus could equally well be used for a calculation based on an expansion as well as a contraction in volume, but it was thought that an increase rather than a decrease in pressure might be less harmful to living tissue.

Dimensions

These depend on the size of objects to be measured and the accuracy required. For objects up to about 4 ml. canisters of about 20 ml. and a pipette of about 10 ml. were found useful. This gives v_2 as $\frac{1}{3}v_1$. If the tube H consists of tubing 2 mm. internal diameter, each centimetre along the tube H represents 0.1 ml. of the object v_1 . These dimensions involve an excess pressure of about 0.5 atmosphere in the apparatus.

Construction

The diagram represents the apparatus in one plane; a more compact arrangement was possible in practice. Connecting tubes were of 1–1.5 mm. capillary tubing, as was the manometer in which Brodie's fluid was used. Rubber connexions were used, but care was taken that the ends of the glass tubes abutted closely together. The canisters were made from B19 universal ground-glass joints. It was essential to clamp these joints tightly since there was a danger of their being loosened by the pressure inside. This was done as shown for canister C_2 . The tubulated stopper was held by a split bung wedged into a hole in the bracket B . The canister was held from below by a rubber bung supported on the clamping plate A . It was advantageous to increase the

heat capacity of the canisters by surrounding them with mercury held in stout specimen tubes (*D*).

The mercury reservoir *R* was clamped to a slide on a vertical brass rod *E*. The slide was also fixed to the continuous chain *F*. The height of the reservoir could be adjusted by manipulating the chain, and a fine adjustment obtained by inserting the peg *G* into a link of the chain and turning the milled head *J*. Distances between h_1 and h_2 were measured by fixing a centimetre scale behind *H*.

Calibration

The canister C_2 was made larger than C_1 and equality of volume attained by adding mercury to C_2 . The quantity of mercury to be added was ascertained as follows. The volumenometer was operated without the addition of mercury and the reading on the scale at h_2 noted. The mercury in *H* was then raised again to h_1 , the zero position, a known volume of mercury put into C_2 , and a new reading h_2^1 ascertained in a second operation. From the difference between these two readings h_2 and h_2^1 , the volume of mercury to be added to C_2 to make no change in the zero reading necessary was calculated by simple proportion. The final adjustment in volume between the canisters was, in fact, made by varying the position of the zero reading h_1 until an injection of V_2 into each canister resulted in no pressure difference being registered by the manometer.

In order to calibrate the scale along *H*, known weights of mercury were put into C_1 and the corresponding readings of *H* noted. The following calibration results were obtained. The volumes of mercury were calculated from the weights taken.

	Vol. of mercury taken (ml.)	Volumenometer reading (mean of 3) cm.	Calibration factor (ml./cm.)
(1)	0.890	7.25	0.1228
(2)	1.793	14.55	0.1232
(3)	2.974	24.30	0.1224
(4)	4.221	34.23	0.1233

Mean factor for apparatus 0.1229 ml./cm.

Manipulation

The object is placed in C_1 and the taps T_1 , T_2 , and T_3 opened. The mercury level in *P* is adjusted exactly to p_1 using *J*. Taps T_2 and T_3 are then closed and with T_4 communicating with C_2 , the mercury level is raised to p_2 . This compression should be slow and is best done by closing T_5 , raising *R*, and then opening T_5 until a slow stream of mercury fills the pipette. T_5 is then closed, *R* lowered, and T_4 turned to communicate with C_1 . T_5 is now opened and the pipette allowed to fill with air, after which T_1 is closed and volume V_2 is compressed into C_1 and T_4 closed. T_3 is now opened, but if the volume of the object is considerable, it is necessary to lower the mercury in *H* before opening T_3 or the Brodie fluid may get blown out of the manometer. The mercury level in *H* is adjusted by connecting it with *R* through T_5 , and varying the

height of R , once again using the fine adjustment $\mathcal{F}$, until the levels of the fluid in the manometer are the same. The reading in H now gives a measure of the volume of the object. A few minutes should be allowed for equilibrium to be attained before making the final adjustment.

Example of use of volumenometer

Measurement of intercellular space volume of plant tissues.

If the intercellular space system of a tissue is continuous with the surrounding atmosphere—as in disks of leaf or storage tissue—the volumenometer will measure the volume of *cells* in the sample. A liquid displacement method, e.g. pycnometer, measures the volume of the *tissue*. The difference between the values given by these two methods therefore measures the volume of the intercellular spaces.

1. *Potato*. Cylinders about 1.25 cm. diam. were cut with a cork-borer and carefully dried.

	Sample	
	1	2
Tissue volume (ml.)	3.915	4.318
Cell volume (ml.)	3.773	4.179
Vol. of intercellular spaces (ml.)	0.142	0.139
% intercellular space in tissue	3.6%	3.2%

2. *Iris leaf*. Disks of tissue about 1.25 cm. diam. punched with cork-borer.

	Sample	
	1	2
Tissue volume (ml.)	1.578	2.543
Cell volume (ml.)	1.172	1.930
Intercellular space volume (ml.)	0.406	0.613
% intercellular space in tissue	25.7%	24.1%

It is suggested that this form of volumenometer might find use in measuring the volumes of germinating seeds or bulky plant organs prior to use in Warburg respirometers. Also the pore space of a soil sample occupied by air could readily be determined. By altering the dimensions of the apparatus equal accuracy could be obtained in measuring very small volumes which might find application in growth studies.

The Development of Cells in the Growing Zones of the Root

R. BROWN AND D. BROADBENT

Botany Department, University, Leeds

INTRODUCTION

THE investigation described below represents a study in the development of cells from the meristematic to the fully extended state in roots that are growing normally from germinated seeds. The process has been investigated with particular reference to cell volume, dry weight, protein content, and respiration. Volume and dry weight changes have been examined since they are measures of the overall growth of the cell. Dry weight has the further significance that from it, when the total nitrogen content is known, an estimate may be obtained of the weight of the wall. Protein and respiration changes have been determined since they indicate changes respectively in the mass and the metabolic activity of the protoplast.

The general technique used has been to take serial sections 0.2–0.6 mm. thick along the course of a root from the apex towards the base and to determine on different but corresponding groups of sections the number of cells they contain, total and soluble nitrogen, dry weight, and respiration. From these data the measurement per cell is obtained by dividing the appropriate figure for the section by the number of cells it contains. This procedure yields a figure which is an average measure for the cells in any particular zone of the root. The volume data show, what is evident from normal morphological examination, that the volume of the cell increases with increasing distance from the apex, and corresponding measurements of protein and respiration at different distances therefore refer to cells of different sizes. It may be emphasized, however, that the figures obtained are averages for particular zones and do not represent data relevant to any particular cell type. In any zone the sizes of the cells vary considerably, and for the limited purpose of measuring growth techniques such as those of Brumfield (1942) and Burstrom (1941), which depend on the observation of single cells, may be preferable. On the other hand, with these techniques it is difficult, if not impossible, to relate the linear measurements to measurements of other aspects of growth.

MATERIALS AND METHODS

The observations have all been made on roots of peas which have been obtained by germinating seeds in sand with a constant moisture content, the cultures being maintained in the dark at a constant temperature of 25° C. for 64 hours. The material for the investigation has always been taken from roots 2–3 cm. in length. It has been found that the lengths of the meristematic and

extending zones vary according to the age of the root, and the use of roots of constant age and of approximately constant length is therefore an important experimental condition.

Sectioning technique. Each observation recorded below has been made with a large number of sections, and an apparatus has been devised for obtaining serial sections from a number of roots simultaneously. The apparatus consists in principle of eight vertical rods attached to a horizontal platform and accommodated in eight channels running through two superimposed metal blocks. The platform to which the rods are attached is supported on a screw which traverses a supporting metal disk, and which is provided at its lower end with a large head, the edge of which is notched. The notches engage with a ratchet, and the number of these that pass the ratchet when the head is rotated is a measure of the distance through which the screw and the rods are lifted.

When the apparatus is used the rods, the upper ends of which are all at the same level, are brought to the upper surface of the lower metal block. The upper block, being detachable, is removed and eight roots are then pushed through the channels until their tips are level with the upper surface, after which a razor is drawn over the lower surface, giving eight roots of exactly the same length in position in the block, which is then inverted and secured in position above the first block. When in position the channels of the upper block correspond exactly with those of the lower, and the cut surfaces of the roots rest on the upper ends of the rods. The screw head is now turned to bring the tips of the roots a known distance above the upper surface of the upper block. A razor is drawn across the free surface, excising eight sections of known thickness. The process is repeated until the required number of groups of serial sections has been obtained.

With this apparatus eight sections from the same zone are obtained simultaneously. With sections taken from maturer parts of the root the sample is relatively uniform. Towards the apex, however, the root is conical, and it may not be rigidly supported against the side of the channel. As a result, in the excision of the section the cutting may be oblique and samples of terminal sections may not be uniform. This is particularly the case with samples of the first sections of the series.

Normally each observation requires considerably more than eight sections. When a group of eight roots has been sectioned to the desired level it is replaced by another group and the process repeated until samples of the requisite size have been obtained. With this technique samples of 150 sections for 10 successive zones each 0.2 mm. thick can be obtained in 3 hours. Each series of observations is made with serial sections over the first 9.0 mm. from the apex. Some observations have been made with sections 0.2 mm. thick, but it has been found that accurate cutting with sections of this size is only possible towards the apex of the root. With zones that consist entirely of vacuolated cells the tissues are not sufficiently rigid to withstand thin sectioning with the technique used. In these zones, therefore, only sections 0.4 mm. or more thick have been excised.

Determination of cell number. The total number of cells in a group of sections has been determined by the maceration technique described by Brown and Rickless (1949). An important modification of the method, however, has been applied in the present connexion. The earlier workers found that with root tips of *Cucurbita* that had been immersed in 5 per cent. chromic acid for 24 hours, suitable maceration was obtained by shaking the fluid containing the tips by hand. With pea root tissue, however, the separation of the cells has required forcing the suspension through a fine jet at the end of a pipette. The suspension is repeatedly drawn into and expelled from the pipette under pressure.

The observations on cell numbers have been made in the apical region on sections 0.2 mm. thick, and 30 sections have been used for each observation.

Determination of cell volume. The average cell volume has been determined by dividing the volume of the section by the number of cells it contains. The volume of the section is calculated from the diameter and the known thickness of the section. In this estimate of the average volume no provision is made for the volume occupied by the intercellular space system. The area covered by intercellular spaces in sections is not greater than 10 per cent., and the error involved in the estimate of the average volume is therefore not likely to be greater than this amount. Further, this error is clearly negligible since the volume of the cells increases at least thirtyfold.

Determination of dry weight. For the dry weight determination 150 sections for each zone, each section being 0.2 or 0.4 mm. thick, are placed in a small weighing bottle, heated for 3 hours at 80° C., and then weighed. The accuracy of the method has been examined by comparing results obtained with the standard technique with others obtained by a freeze-drying method and it has been found that results from the two methods do not differ significantly.

Determination of total and soluble nitrogen. For this purpose a sample of sections is ground to a fine paste, which is made into a suspension of known volume. Total nitrogen is determined on an aliquot and soluble nitrogen on a further portion after precipitation of the protein with 2.5 per cent. trichloroacetic acid. The nitrogen contents of the different fractions have been determined with a microkjeldahl technique, metallic selenium being used as a catalyst during the digestion with sulphuric acid. Protein nitrogen is estimated by deducting soluble from total nitrogen. This procedure is based on that used by Richards and Templeman (1936).

In this connexion sections 0.6 mm. thick have been used, and 150 sections have been included in the sample for each observation. It has been found that the time required to assemble enough material with sections less than 0.6 mm. thick is too great to ensure a uniform sample.

Determination of respiration rate. This has been measured manometrically in a Barcroft apparatus. During the experiment the tissue is suspended in water. The rate of oxygen absorption is measured over a period of 3 hours, during which it remains constant. In some instances the rate of carbon dioxide production has also been measured, again over a period of 3 hours.

The measurement of the respiration rate in different zones has presented several difficulties which are not involved in the other observations of the investigation. Clearly a uniform sample of sections must be obtained for each determination. Uniformity, however, cannot be ensured if the time involved in assembling the sample is large, and in order to obtain sufficient material rapidly it has been necessary to use sections which are not less than 0.4 mm. thick. Again, the thickness of the sections must be such that the respiration of the tissue is not affected by the sectioning procedure. It has been found that within the first 4.0 mm. from the apex the combined respiration rates of the successive sections, each 0.4 mm. thick, does not differ significantly from the intact fragment. Also, the respiratory quotients of the intact fragments and of the different sections are all between 0.90 and 1.0. With the mature zone, on the other hand, sections 0.4 mm. thick have a lower respiration rate than the corresponding tissue in the intact root, and in the zone 4.0–9.0 mm., therefore, sections 0.8 mm. thick have been used. It is probable that when the cells are large and fully vacuolated sectioning involves the destruction of an appreciable proportion of the total number of cells, and the combined respiration rates of these sections therefore depend on a smaller number of cells than in the case of the corresponding intact tissue.

Finally, although with dry weight and protein content duplicate determinations agree closely, with respiration they do not, and replicates may differ by as much as 40 per cent. The values given in the next section for the regions near the tip where the variation is greatest are, therefore, the means of two or three replicate determinations.

As indicated, sections 0.4 and 0.8 mm. thick have been used for respiration measurements. For each observation 150 sections have been used.

RESULTS

The data with regard to volume, dry weight, protein content, and respiration are presented separately below. In each case, except volume, two sets of data are given, namely, values reduced to a unit section basis, and values reduced to a unit cell basis.

For reasons that are given above the sections used in the different determinations have varied in thickness. The results per section, however, are all presented as for a section 0.4 mm. thick. With volume and dry weight determinations the observations towards the apex of the root have been made with sections 0.2 mm. thick, and the values for a 0.4-mm. section have been obtained by adding together the results of determinations on successive sections. The data for respiration have not involved any conversion since the observations are based on 0.4-mm. sections. With protein nitrogen, which has been determined with 0.6-mm. sections, values for 0.4-mm. units have been calculated by assigning two-thirds of the experimental value to a 0.4-mm. zone when this is covered by a 0.6-mm. section, and assigning a third of each of two successive experimental values to a 0.4-mm. zone when this

corresponds to the lower third of one section and to the upper third of the next.

Cell volume. The change in cell volume with increasing distance from the apex is shown by the data of Table I and Fig. 1. It is evident that the volume of the cell increases progressively with increase in distance from the apex of the root over the first 4.8 mm. At that point there is a change in the situation and thereafter volume changes little, if at all, with increasing distance. It may be noted that the average increase is about thirtyfold.

TABLE I

Numbers of cells, NC, in sections and average volumes of cells, V/C , in sections ($\text{cm.}^3 \times 10^{-3}$) at increasing distances from apex of the root

Distance (mm.)	NC	V/C
0.0-0.4	15,800	6.8
0.4-0.8	35,600	8.7
0.8-1.2	38,200	10.8
1.2-1.6	32,400	15.3
1.6-2.0	22,400	25.4
2.0-2.4	19,100	34.7
2.4-2.8	14,400	47.9
2.8-3.2	13,300	53.3
3.2-3.6	9,400	90.8
3.6-4.0	6,800	113.5
4.0-4.4	5,360	164.0
4.4-4.8	4,900	179.6
4.8-5.2	4,900	179.6
5.2-5.6	5,080	173.2
5.6-6.0	5,200	169.2
6.0-6.4	5,100	172.5
6.4-6.8	4,900	179.6
6.8-7.2	5,200	169.2
7.2-7.6	4,900	179.6
7.6-8.0	4,600	193.3
8.0-8.4	4,800	183.3
8.4-8.8	—	—
8.8-9.2	4,600	191.3

The average volume per cell has been calculated in the same way as all the other values that refer to the unit cell. The value for the appropriate characteristic of the section has been divided by the number of cells it contains. The data showing the number of cells in successive sections are therefore of some significance for the investigation as a whole and may be considered in this connexion. The data of Table I and Fig. 2 show the number of cells in successive sections 0.4 mm. thick out over a distance of 9.0 mm. from the apex. The number increases from the apex to about 1.2 mm., decreases from this point to about 4.8 mm., and remains more or less constant between 4.8 and about 9.0 mm. The root is conical over about the first two millimetres, and the increase in numbers over the first 1.2 mm. is no doubt due primarily to this condition, although the decreasing proportion of root-cap cells in each successive section probably contributes to it. Between 1.2 and 4.0 mm. the effect of

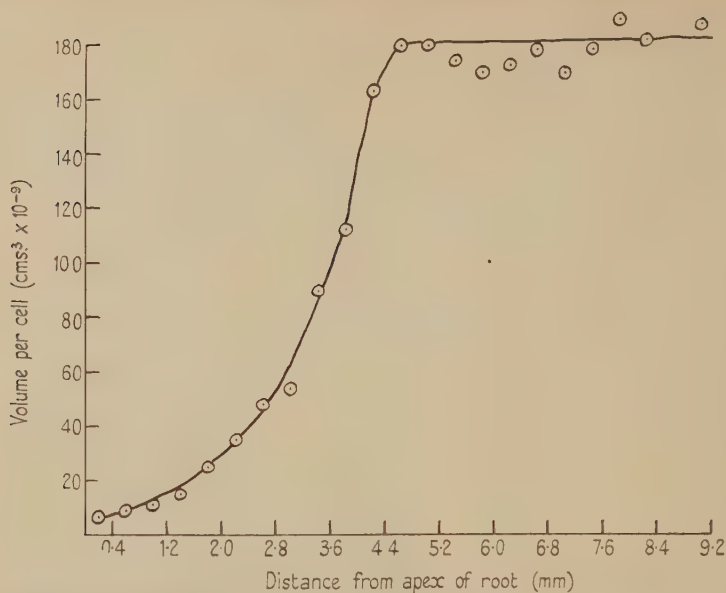


FIG. 1. Average volume per cell at increasing distances from the apex of the root.

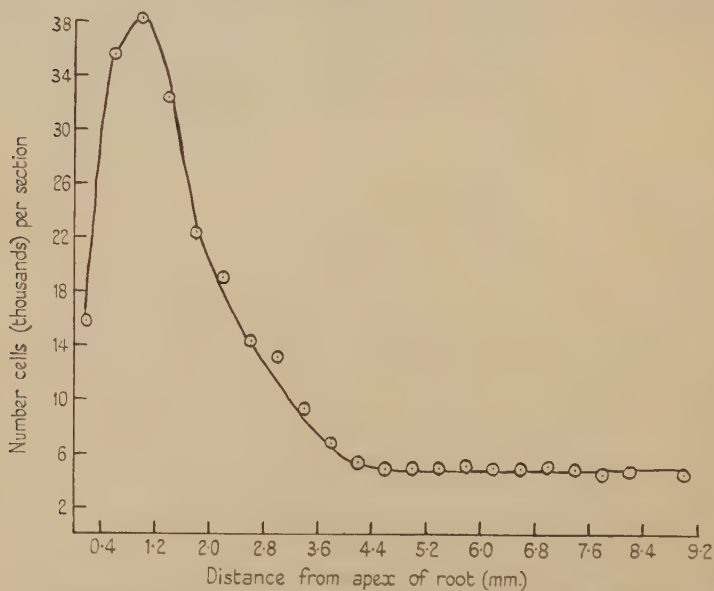


FIG. 2. Numbers of cells in successive sections from the apex of the root. Each section 0.4 mm. thick.

TABLE II

Dry weight per section, DW/S ($\text{g.} \times 10^{-6}$), and per average cell in the section, DW/C ($\text{g.} \times 10^{-10}$), at increasing distances from the apex of the root

Distance (mm.)	DW/S	DW/C
0.0-0.4	18.7	11.8
0.4-0.8	22.8	6.4
0.8-1.2	30.6	8.0
1.2-1.6	39.5	12.2
1.6-2.0	45.6	20.4
2.0-2.4	47.7	25.0
2.4-2.8	42.8	29.7
2.8-3.2	45.6	34.3
3.2-3.6	38.0	40.4
3.6-4.0	36.7	54.0
4.0-4.4	32.4	60.4
4.4-4.8	30.6	62.4
4.8-5.2	28.4	58.0
5.2-5.6	29.2	57.5
5.6-6.0	32.4	62.3
6.0-6.4	32.0	62.7
6.4-6.8	31.4	64.1
7.6-8.0	32.0	69.6
8.8-9.2	32.4	70.4

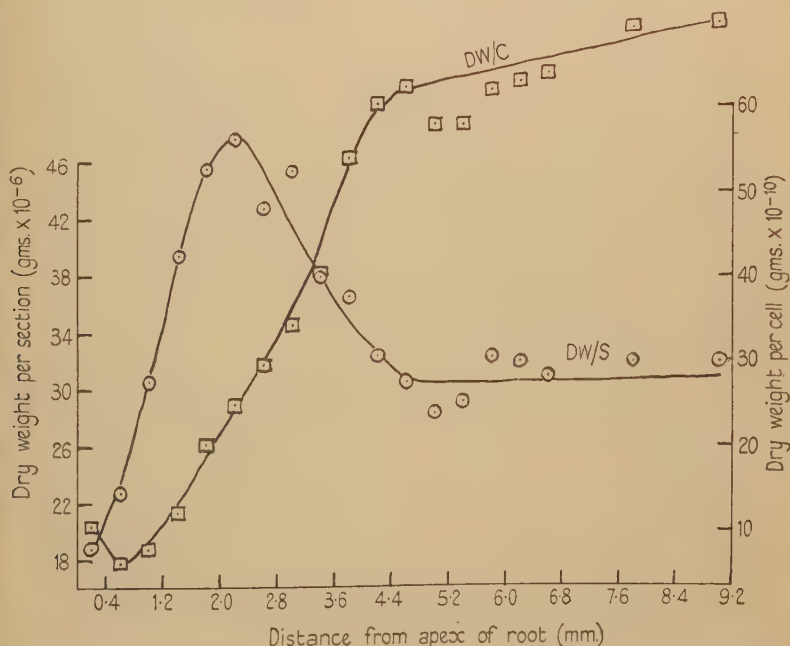


FIG. 3. Dry weight of sections (DW/S) and of average cells in the sections (DW/C) at increasing distances from the apex of the root.

TABLE III

Protein nitrogen per section, PN/S ($g. \times 10^{-6}$), and per average cell in the section, PN/C ($g. \times 10^{-10}$), at increasing distances from the apex of the root

Distance (mm.)	PN/S	PN/C
0.0-0.4	0.44	0.30
0.4-0.8	1.88	0.53
0.8-1.2	3.44	0.96
1.2-1.6	3.55	1.13
1.6-2.0	3.66	1.63
2.0-2.4	3.02	1.58
2.4-2.8	2.79	1.93
2.8-3.2	2.56	1.93
3.2-3.6	1.54	1.66
3.6-4.0	1.52	2.40
4.0-4.4	1.55	3.15
4.4-4.8	1.58	3.33
4.8-5.2	1.58	3.43
5.2-5.6	1.54	3.01
5.6-6.0	1.50	2.90
6.0-6.4	1.36	2.77
7.6-8.0	1.14	2.52
8.8-9.2	1.14	2.52

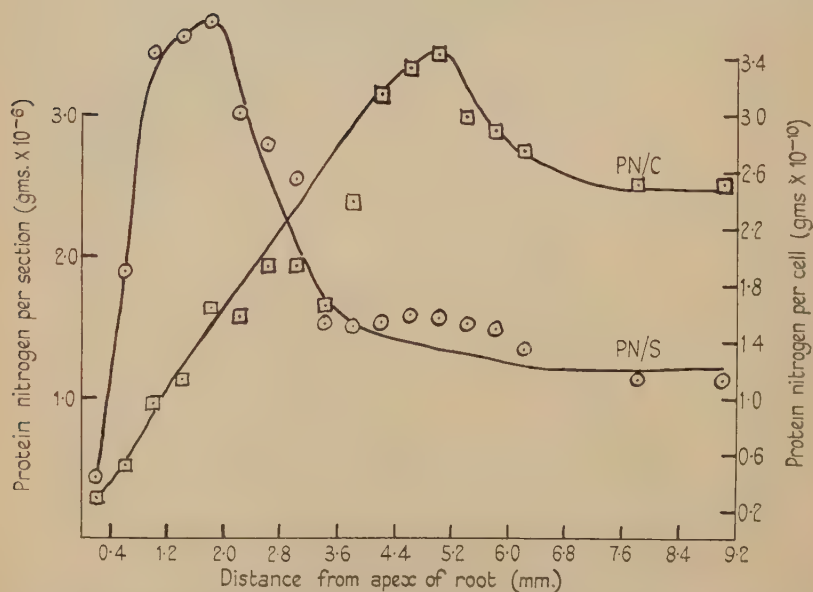


FIG. 4. Protein nitrogen content of sections (PN/S) and average cells in sections (PN/C) at increasing distances from the apex of the root.

TABLE IV

Respiration per section, R/S ($\mu\text{L} \times 10^{-1} \text{O}_2/\text{hr.}$), and per average cell in the section, R/C ($\mu\text{L} \times 10^{-6} \text{O}_2/\text{hr.}$), at increasing distances from the apex of the root

Distance (mm.)	R/S	R/C
0.0-0.4	1.4	8.9
0.4-0.8	3.0	8.4
0.8-1.2	4.5	11.9
1.2-1.6	5.1	15.4
1.6-2.0	4.5	19.9
2.0-2.4	4.4	23.0
2.4-2.8	4.0	27.8
2.8-3.2	3.1	23.1
3.2-3.6	2.9	30.9
3.6-4.0	3.0	44.1
4.0-4.4	2.9	54.5
4.4-4.8	2.9	59.2
5.2-5.6	2.3	45.3
6.0-6.4	2.6	51.0
6.8-7.2	2.1	40.8
7.6-8.0	2.2	45.8
8.4-8.8	2.2	47.8

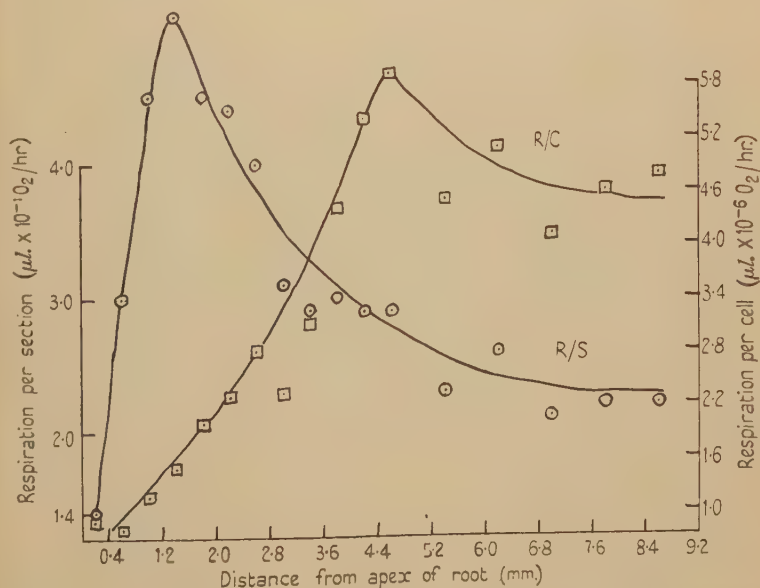


FIG. 5. Respiration of sections (R/S) and of average cells in sections (R/C) at increasing distances from the apex of the root.

the increasing diameter of the root does not compensate for the increasing size of the cells, and the number of cells in successive sections therefore decreases.

Dry weight. The dry weights of successive sections each 0.4 mm. thick and the dry weights of the average cell in successive sections are shown by the data of Table II and Fig. 3.

The dry weight per section from the tip to about 2.4 mm. increases. From 2.4 to 4.8 mm. it decreases and from 4.8 to about 8.0 mm. it remains more or less constant. The initial increase is no doubt again related to the conical form of the root, and the decrease to the increasing volumes of the individual cells.

The dry weight per cell increases from the tip to about 4.8 mm., and from this point to 9.0 mm. it either remains constant or only increases gradually. From the apex to about 4.8 mm. there is about a tenfold increase in dry weight.

Protein nitrogen. The data for protein nitrogen in successive sections and in the average cells that these sections contain are shown in Table III and Fig. 4.

Protein nitrogen per section increases from the tip to about 2.0 mm., decreases from this point to about 3.6 mm., and with further increase in distance remains constant. The initial rise may again be related to the conical form of the root and the decrease to the increasing volumes of the cells.

The protein content per cell increases uniformly from the apex to about 5.2 mm., and then decreases slightly from this point to about 6.8 mm. and remains more or less constant with further increase in distance. From the apex to about 5.2 mm. there is about a tenfold increase in protein content of the cell.

Respiration. The respiration rates of successive sections and of the average cells that they contain are shown by the data of Table IV and Fig. 5.

The respiration per section increases from the apex to about 1.5 mm., decreases from this point to about 5.6 mm., and remains constant from 5.6 to about 9.0 mm. Similar data to these have been published by a number of earlier workers. Gregory and Woodford (1939) showed that the terminal zone of 10 mm. in the root of bean has a higher respiration rate than more mature tissue. Machlis (1944) showed with roots of barley that the respiration rate of millimetre zones decreased from the apex to about 8.0 mm. from it. Berry and Brock (1946) working with onions, and Wanner and Leupold (1947) working with maize showed that the zone of maximum respiration is not at the apex but immediately behind it. The same conclusion is shown by the present series of data, and this increase is undoubtedly an effect of the increasing diameter of successive sections over the first 1.6 mm.

The present results, however, also show that the respiration per cell increases from the apex to about 4.8 mm., decreases slightly from 4.8 to about 6.0 mm., and from this point to about 9.0 mm. remains more or less constant.

DISCUSSION

It is an important feature of this series of observations that they cover the development of the cell from the meristematic to the fully vacuolated state. Anatomical observation shows that vacuolated cells are only present in regions

more remote from the apex than about 1.5 mm. Within the first 1.5-mm. zone the cells are all meristematic except for root-cap cells, which are restricted to about the first 0.4 mm. Thus the first four sections, each 0.4 mm. thick in the experimental series, consist entirely of meristematic cells. All succeeding sections in the series contain almost exclusively vacuolated cells.

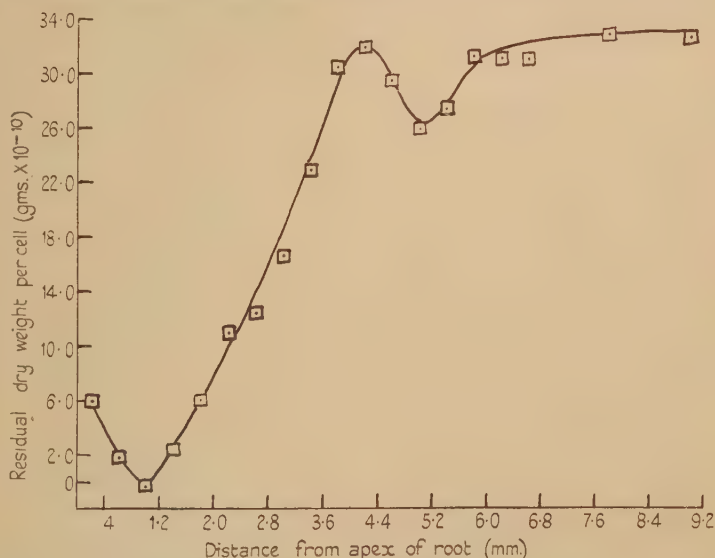


FIG. 6. Residual dry weight of average cells at increasing distances from the apex of the root.

It is evident from the data of Fig. 1 that the volume of the cell increases throughout the meristematic zone, and continues to increase until about 4.8 mm. from the apex. Similarly with dry weight, protein content, respiration—they all increase from the apex to about 4.8 or 5.0 mm. Clearly, as extension proceeds dry weight, protein content, and respiration all increase with increasing volume of the cell. A similar conclusion has been reached by Kopp (1948) with respect to protein and respiration. Further, the increases that occur after vacuolation are continuations of corresponding increases that proceed while the volume of the cell is increasing in the meristematic stage.

Other properties of the cell, however, also change as extension occurs. The data of Fig. 6 show the dry weight per cell for components other than those of the total nitrogen fraction. These data have been obtained by multiplying total nitrogen by the conventional factor of 6.25 and deducting this value from the total dry weight of the cell. Clearly this residual dry weight is due to a variety of cell components, but the largest fraction in it is undoubtedly the cell wall, and it may therefore be taken as a crude estimate of this component. It is evident that in the root the weight of the wall increases as extension proceeds, and the position is similar to that already described for the coleoptile by

Bonner (1933) and by Wirth (1946). Two further aspects of the position may, however, be noticed. In the meristem the polysaccharide content per cell is clearly negligible; secondly, that when extension ceases there is a decrease in dry weight followed by a recovery. The decrease may not be due to a corresponding change in wall weight but may be the result of a loss of solutes from the cell. The recovery, on the other hand, may represent an increase in the thickness of the wall.

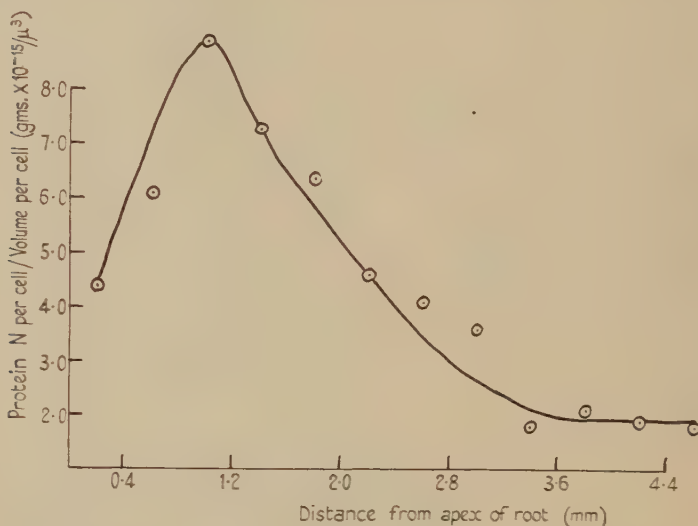


FIG. 7. Ratios between protein nitrogen content and volume of the cell.

The increase in protein content during extension corresponds to an earlier observation of Blank and Frey-Wyssling (1944) who showed the same condition in extending cells of the hypanthium of *Oenothera*. It may be emphasized in this connexion that protein content increases throughout the course of development up to the fully vacuolated state, and the meristematic cell therefore has a considerably lower protein content than the mature cell. A further aspect of the protein content is shown by the data of Fig. 7, which are ratios between protein content and volume of the cell, and these values have been plotted against distance from the apex of the root in the region over which extension is continuing. Since the cells grow as cylinders, surface in this case is directly proportional to volume. The ratios decrease from a point in the meristematic region to about 5.0 mm. from the apex. This decrease suggests that as extension proceeds the amount of protein and therefore of the protoplast decreases per unit area of cell surface. This is a feature of the cell which may be of some importance in relation to the rate and period of extension.

Brown and Sutcliffe (1950) have already shown that when excised extending root tissue is suspended in sugar solution respiration increases as the

length of the cell increases. The present series of data show that respiration also increases when extension occurs in the intact seedling root. This increase continues over the whole range of development up to the fully vacuolated state, and the non-vacuolated meristematic cell therefore has a considerably lower respiration rate than the mature cell.

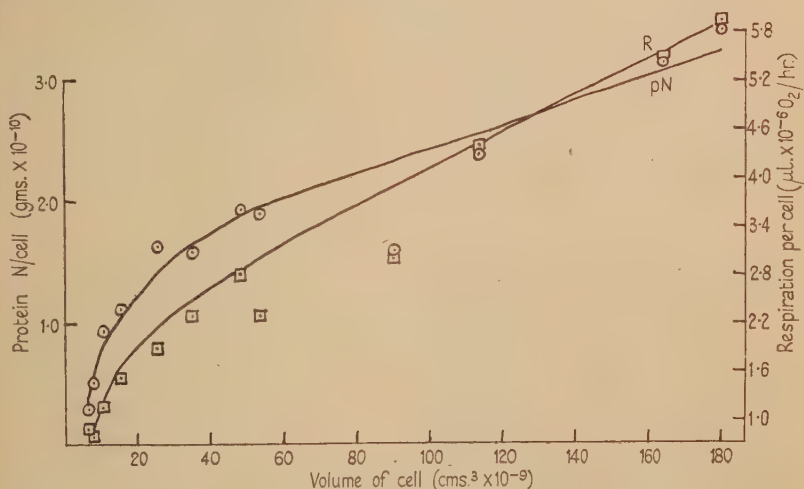


FIG. 8. Protein nitrogen content and respiration plotted against corresponding cell volumes.

It may be emphasized that this low respiration rate is not due to the development of anaerobic conditions in the meristem, since, as explained above, the respiration of the section from the meristem is not greater than that of the corresponding zone in the intact root, and the respiratory quotient of the intact tissue and of the section is probably approximately 1.0.

With both protein content and respiration there is a slight but significant decrease per cell immediately extension ceases, and the change with respect to these two aspects suggests that when extension ceases there is a change in the metabolic situation. Brown and Sutcliffe (1950) have also shown that with excised tissues growing in sugar solutions the respiration rate also decreases when extension ceases.

Clearly there is a close connexion between protein content and respiration during extension; both increase during this phase of growth and both decrease when growth ceases. The connexion, however, is further emphasized by curves of Fig. 8, in which the respiration and protein contents have been plotted against the corresponding volumes for the phase in which growth is occurring. With both in the early phase of extension the increase is large for unit increment in volume, but with both in the later phase the increase is relatively smaller. Further, if there is a simple direct dependence of the one on the other, then the respiration per unit protein should be constant. The

relevant ratios are given in Table V. These values suggest that although the ratios are larger for the mature than they are for the meristematic cell, the difference, if it is a real one, is not large.

TABLE V
Respiration per unit protein N
Distance from apex
of root (mm.) $\mu\text{l. O}_2/\text{mg. N/hr.}$

0.0-0.4	290
0.4-0.8	160
0.8-1.2	160
1.2-1.6	140
1.6-2.0	120
2.0-2.4	140
2.4-2.8	140
2.8-3.2	120
3.2-3.6	190
3.6-4.0	190
4.0-4.4	170
4.4-4.8	180
4.8-5.2	180
5.2-5.6	150
5.6-6.0	150
6.0-6.4	190
7.2-7.6	180
8.4-8.8	190

The curves of Fig. 8 suggest that there are two phases in the development of the cell. Burstrom (1941, 1942) has also provided evidence that shows that with respect to the properties of the wall and of the reaction of the cell to nutrient and temperature treatments two phases are again involved. The phases of Burstrom may correspond to those found here, although the identity requires to be confirmed by further investigation. It may be noted that the phases shown by the curves of Fig. 8 do not correspond to the meristematic and extending phases, since although the values of the first phase include those obtained in the meristematic region, they also include some which refer to zones which are some distance away from it.

Thus, with respect to the development of the cell in the root from the meristematic to the fully vacuolated state, the present series of data indicate that at least three phases are involved. There are, firstly, an early phase in extension in which unit change in volume is accompanied by large increases in protein content and respiration; secondly, a later phase in which the increases are relatively smaller; and finally, a phase which sets in when extension ceases, when respiration and protein content decrease, and when there may be a change in the permeability of the protoplast to solutes in the vacuole.

SUMMARY

1. Data are presented which show the change in volume, dry weight, respiration, and protein content of the cell during development from the meristematic to the mature fully extended state.

2. The data have been obtained by making serial sections of known thickness along the root, making the relevant observations on the sections, and relating the values obtained to the total number of cells in the sections. An apparatus is described for obtaining serial sections from a group of roots.

3. It is shown that the weight of the wall, the protein content, and respiration all increase during extension, and the increases during active growth with respect to respiration and protein content occur in two phases. In the first the increases are large per unit increment in volume; in the second they are relatively smaller. When extension ceases a third phase in the development of the cell sets in in which respiration and protein content decrease slightly.

4. The data show that the protein content and the respiration in the meristematic cell are considerably lower than they are in the fully vacuolated cell.

LITERATURE CITED

- BERRY, J. I., and BROCK, M. J. (1946). Polar distribution of respiratory rate in the onion root tip. *Plant Physiol.* **21**, 542.
- BLANK, F., and FREY-WYSSLING, A. (1944). Protoplasmic growth in the hypanthium of *Oenothera acaulis* during cell elongation. *Ann. Bot.*, N.S. **8**, 71.
- BONNER, J. (1933). The action of the plant growth hormone. *J. Gen. Physiol.* **17**, 63.
- BROWN, R., and RICKLESS, P. (1949). A new method for the study of cell division and cell extension with preliminary observations on the effect of temperature and nutrients. *Proc. Roy. Soc., B*, **136**, 110.
- and SUTCLIFFE, J. F. (1950). The effects of sugar and potassium on extension growth in the root. *J. Exp. Bot.* **1**, 88.
- BRUMFIELD, R. T. (1942). Cell growth and division in living root meristems. *Amer. J. Bot.* **29**, 533.
- BURSTROM, H. (1941a). Studies on the carbohydrate nutrition of roots. *Ann. Agric. Coll. Sweden*, **9**, 264.
- (1941b). Formative effects of carbohydrate on root growth. *Bot. Notiser*, 310.
- (1942). Influence of heteroauxin on cell growth and root development. *Ann. Agric. Coll. Sweden*, **9**, 264.
- GREGORY, F. G., and WOODFORD, E. K. (1939). Apparatus for the study of the oxygen, salt and water uptake of various zones of the root. *Ann. Bot.*, N.S. **3**, 147.
- KOPP, M. (1948). Über das Sauerstoffbedürfnis wachsender Pflanzenzellen. *Ber. Schweiz. bot. Ges.* **58**, 283.
- MACHLIS, L. (1944). The respiratory gradient in barley roots. *Amer. J. Bot.* **31**, 281.
- RICHARDS, F. J., and TEMPLEMAN, W. G. (1936). Physiological studies in plant nutrition. IV. Nitrogen metabolism in relation to nutrient deficiency and age in leaves of barley. *Ann. Bot.* **50**, 367.
- WANNER, H., and LEUPOLD, U. (1947). Über die longitudinale Verteilung der Saccharaseaktivität in der Wurzelspitze. *Ber. Schweiz. bot. Ges.* **57**, 156.
- WIRTH, D. (1946). Membranwachstum während der Zellstreckung. *Ber. Schweiz. bot. Ges.* **56**, 175.

Studies in Abscission

I. FACTORS AFFECTING THE INHIBITION OF ABSCISSION BY SYNTHETIC GROWTH-SUBSTANCES

H. W. B. BARLOW¹

*Research Institute of Plant Physiology, Imperial College of
Science and Technology, London*

INTRODUCTION

THE fact that certain synthetic growth-substances inhibit abscission has been extensively applied to the control of 'preharvest drop' in several varieties of apples and pears, and the range is being steadily widened both of compounds which are effective and of fruit varieties which respond. Nevertheless, little is known about the mode of action of these synthetic growth-substances, or, indeed, of the process of abscission itself and its natural control by the plant, and the present studies are being undertaken to gain a better understanding of these problems.

Abscission is a widespread phenomenon in plants, and has been defined by Lloyd (1927) as 'a mode of activity of living cells whereby they separate from the tissue in which they occur'.

Apart from the familiar shedding of leaves by deciduous trees, and of flowers and fruits of many kinds, there are numerous less obvious examples of abscission in other organs: in the stem abscission may occur at a node (e.g. *Impatiens* (Lloyd, 1914) and *Mirabilis* (Lloyd, 1916; Beal and Whiting, 1945)) or in an internode (e.g. *Phaseolus* (Whiting and Murray, 1948)); in inflorescences at the base, as in many catkins, or elsewhere, as in the spurs of apples and pears under certain circumstances; in the flower, shedding of the corolla is a typical abscission process, as is the separation of individual achenes in compound fruit (Ulrich, 1944), while in the 'shot-hole disease' of *Prunus* spp. parts of the leaf lamina itself are cut off by an abscission process (Samuel, 1927).

The separation of parts of algae is also due to the same process (Lloyd, 1927).

A later paper in this series will deal with the anatomy of the abscission process, so here it will suffice to mention that the separation of the plant part concerned is ultimately due to the dissolution of the middle lamella, and sometimes other parts of the cell wall, in a limited region of the plant tissues. This region may be one in which the cells show no particular organization, as in the mango and avocado, where the ripe fruit is merely torn from the stalk by its own weight, following changes in the cell walls similar to those observed

¹ The work was carried out at East Malling Research Station.

in the ripening fruit flesh (Barnell, 1939), or they may form an 'abscission zone' of varying complexity, within which the final separation process takes place, as in many leaves (e.g. *Coleus* (Myers, 1940), *Citrus* (Scott *et al.*, 1948)) or in mature apple pedicels (McCown, 1939, 1943; MacDaniels, 1936). In a third class cell-division occurs in a layer or layers of cells across the organ, immediately preceding the separation process, as in stems of *Mirabilis* (Beal and Whiting, 1945) and the immature pedicel of the apple (McCown 1939, 1943; MacDaniels, 1936).

The chemistry of the process has been investigated by Sampson (1918) and McCown (1943), who show that the pectin of the middle lamella breaks down along with the cellulose of the secondary cell wall and sometimes other layers, resulting in lack of cohesion between the cells. Our knowledge of this aspect, however, is still very imperfect.

The connexion between plant hormones and abscission was first suggested in the work of Laibach (1933), who applied orchid pollinia to petioles of *Coleus* from which the lamina had been removed, and found that abscission was thereby prevented for a long time. La Rue (1936) independently extended this work by using lanolin pastes containing among other substances indolylacetic acid, and Myers (1940) followed up with a critical examination of the behaviour of *Coleus* petioles under various experimental treatments.

Gardner, Marth, and Batjer (1939, 1939a) observed a marked effect of α -naphthylacetic acid on the persistence of petiole stubs on cuttings treated with this compound, and this, together with the work of Laibach and La Rue mentioned above, and their own results with holly (Gardner and Marth, 1939) led them to test its effect on the preharvest drop of apples, from the results of which has sprung the widespread commercial application of this and allied substances to control this condition.

The experimental work along this line up till 1945 has been summarized by Vyvyan (1946), since when further work has been mainly on extending the number of varieties of fruit, and the phases of fruit-drop, which are susceptible to control (Vyvyan, 1946; Vyvyan and Barlow, 1947); on searching for other substances which are effective on varieties which do not respond to α -naphthylacetic acid (Batjer and Marth, 1945; Harley, Moon, and Regeimbal, 1947; Gardner and Cooper, 1943); and on various methods of application (Batjer and Thompson, 1948; Marth, Batjer, and Moon, 1945). The number of papers published on these subjects is large, and only a few examples have been given.

In spite of this extensive literature on the control of fruit-drop, relatively little attention has been directed towards the fundamental process of abscission and its natural control by the plant, or to the mode of action of applied synthetic growth-substances, and it is in these fields that the present studies are being made. Early work on abscission itself and various theories as to its cause and control are to be found in Lloyd (1916, 1927) and Heinicke (1919).

MATERIALS AND METHODS

Because fruit-drop control by synthetic growth-substances is of considerable importance, the apple pedicel was chosen as the experimental subject for abscission studies, with the additional advantage that it exhibits two of the main types of the abscission process (with and without cell-division) in its young and mature stages. For certain purposes, however, the changes in its structure during the season make it unsuitable, and apple petioles were therefore used in some experiments.

This paper will deal only with certain factors affecting the inhibition of abscission by synthetic growth-substances and the methods employed for their investigation; other techniques which have been developed for the examination of other factors will be described later in this series.

Variety. The variety Cox's Orange Pippin has been employed in the majority of the experiments, though Bramley's Seedling and Worcester Pearmain have also been used occasionally.

Growth-Substances. Two synthetic growth-substances only were used in these experiments: α -naphthylacetic acid or its sodium salt (referred to subsequently as NAA and NAA Na-salt), and 2:4-dichlorophenoxyacetic acid or its sodium salt (DPOA and DPOA Na-salt.)

General method. The experimental method throughout depends upon the fact that abscission of the apple pedicel or petiole follows the removal of the terminal organ—flower, fruitlet, fruit, or leaf-lamina—and that this abscission is delayed by an appropriate application of growth-substance.

Experimental layout. Ten replicates of each treatment were usually made, arranged in randomized blocks round one tree where possible, though occasionally two trees had to be employed.

'King fruit' pedicels (the terminal fruit of a truss) and any of at all an abnormal nature were avoided.

Observation. After treatment the pedicels were observed at intervals, a light standardized touch being given to each stalk to dislodge it if abscission had taken place, as their own weight was too small to rupture the xylem strands. This standard was attained by using a small piece of spring wire in a holder, and applying a lateral pressure to the pedicel until the wire was bent back to a fixed point on the handle.

Expression of results. Results are expressed as the geometric mean of the persistence in days of the individual replicates of a treatment.

Application of growth-substance. Two quantitative methods of application have been devised, referred to as the 'lanolin emulsion technique' and the 'agar cylinder technique'. The former has been briefly described already (Barlow, 1948), but both will be given in detail here.

Lanolin emulsion technique

A lanolin emulsion prepared according to Winklepleck and McClintock (1941) was found to be much easier to handle than plain lanolin, and had no deleterious effects on the plant.

A stock paste containing the growth-substance was made up by dissolving the acid in the minimum amount of absolute alcohol, or the sodium salt in water, and stirring the solution into the warmed emulsion; from this, various concentrations were prepared by dilution with further emulsion.

Glass syringes were made by selecting narrow tubing which fitted easily into wider tubing, rounding off one end to seal it and to form a bulge slightly too large to go into the outer tube, and then grinding this down to a close fit. The end of this plunger and the end of the outer tube were ground flat.

The syringes were graduated individually by marking the length in each tube occupied by a given weight of mercury, and dividing the distance between into equal divisions. Each division was equivalent to 20 mg. of lanolin emulsion, and on drawing the paste up to fill the syringe, and then moving the plunger from one mark to the next, this amount of emulsion was extruded as a small cylinder. This was cut off with a 'needle-knife' and applied to the place desired.

Agar cylinder technique

Agar as a carrier has obvious advantages over lanolin emulsion for certain purposes, such as applying extracts of plant material to the pedicel or petiole, but, if unprotected, a small agar block under orchard conditions is either rapidly dried up or dislodged by rain or wind. The following method was devised to overcome these difficulties.

Small tubes were made from cellophane drinking 'straws' by cutting these into lengths of about 12 mm. and sealing one end with a drop of 'durofix' adhesive, which dries to form a thin film. Agar made up at 1.5 per cent. and containing the substance under investigation is melted and drawn up into a glass syringe fitted with a glass plunger. When the agar has set, movement of the plunger extrudes a cylinder of the gel of a diameter to fit easily into the cellophane tubes. A special holder carries the syringe and a magazine of the tubes in accurate alinement. After extruding the agar to a fixed gauge mark so that 40 mg. are pushed into the mouth of the tube, the single turn of a knob cuts the agar cylinder by a rotating blade, drops the syringe from the filling position, brings a 'ramrod' into line and drives this forward to push the agar plug to the bottom of the tube. Reversing the movement of the knob allows the charged tube to be expelled into a container and resets the apparatus for filling the next tube.

The charged tubes are inverted over the cut ends of pedicels, so that the agar is protected from the weather. With young thin pedicels the tubes are too loose a fit to prevent desiccation; in this case a plasticine ring is fitted into the open end of the tube after filling and this makes a satisfactory seal when applied to the pedicel.

RESULTS

Of the various factors affecting the inhibition of abscission by synthetic growth-substances, four will be considered here.

1. Concentration of applied growth-substance.

2. Site of application of growth-substance.
3. Distance of applied growth-substance from the abscission layer.
4. 'Period of reaction' of the pedicel or petiole to applied growth-substance.

1. Concentration of applied growth-substance

A relationship had been established in earlier work (Barlow, unpublished, 1938) between the concentration of β -indolylacetic acid applied to debladed petioles of a *Fuchsia magellanica* hybrid and the period of their persistence, and experiments conducted in 1947 on the apple pedicel with NAA have shown a similar relation. In 1948 DPOA was also used, as it was thought that these two growth-substances behaved rather differently.

Only certain experiments need be described in detail, the majority conforming to the general method using lanolin emulsion containing the growth-substance at concentrations from 0.625 to 1000 p.p.m., applied at the standard rate of 20 mg. emulsion per pedicel on the cut surface of the tip. Fig. 1 shows the results of three such experiments on Cox pedicels and one on Bramley's Seedling in 1947. It will be seen that while there is wide variation in the response, there is a threshold value at something over 1 p.p.m., and thereafter an increase in persistence with concentration. The concentrations in this and other graphs are plotted on a logarithmic scale to provide better spacing between them. Fig. 2 shows the results of an experiment conducted with NAA in 1948, using a new set of lanolin emulsions. Here a linear relation was found between the persistence in days (d) and the concentration of NAA in p.p.m. (c) represented by the equation

$$d = 6.52 + 0.0127c,$$

and the graph shows the observed and the calculated values.

DPOA was also used in this same experiment, and Fig. 3 shows the results. The linear relation in this case was represented by

$$d = 5.14 + 0.0194c.$$

Table I gives the values for both compounds; the difference between the observed and the predicted values for the controls was not significant for NAA but was so for DPOA, the observed value (7.19) being above the predicted (5.14).

TABLE I
*Persistence in days of pedicels treated with NAA and DPOA
at eight concentrations*

Concentration (p.p.m.)	NAA		DPOA	
	Observed	Calculated	Observed	Calculated
0	7.19	6.52	(7.19)	5.14
0.5	6.07	6.53	5.51	5.15
1	6.16	6.53	4.63	5.16
5	6.08	6.58	5.11	5.23
10	6.72	6.65	6.20	5.33
50	7.48	7.15	6.15	6.11
100	9.81	7.79	6.63	7.09
500	10.88	12.87	16.45	14.84
1000	21.51	19.22	22.45	24.54

It will be noticed that the threshold value for effectiveness is much higher for the 1948 NAA than for the 1947, being about 10 instead of about 1 p.p.m., and as in the earlier emulsions the acid had been used and in the later the sodium salt, two experiments were conducted in 1948, (a) to compare the two

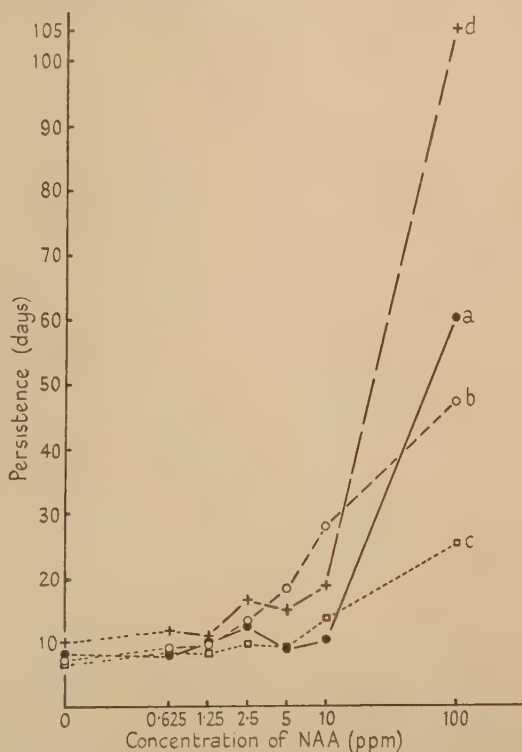


FIG. 1. Relation between concentration of NAA and persistence of defruited pedicels, 1947. Experiments (a-c) on Cox begun on (a) 9 May, (b) and (c) 23 July, (d) on Bramley, 9 May.

sets of emulsions and (b) the acid and its sodium salt. Fig. 4 shows the comparison of the two emulsions and indicates the wide difference between them, which has not been explained. Incidentally it may be noted that the 1947 material had maintained its potency quite well during a year's storage.

Comparison of the acid and its sodium salt over the concentrations 5, 10, 50, and 100 p.p.m. was carried out both for NAA and DPOA with a new set of emulsions, and no significant difference was found between the forms of each substance separately, nor was there any significant interaction between form and concentration. In this experiment the relation between concentration of

DPOA and persistence of the pedicels was again linear and could be represented by the equation

$$d = 10.20 + 0.049c,$$

and the observed value for the controls again differed significantly from the



FIG. 2. Relation between concentration of NAA and persistence of defruited Cox pedicels. Continuous line, observed values; broken line, calculated.

predicted value, though on this occasion it was lower (observed 7.83 days, predicted 10.20 days).

The relation for NAA, however, could not be represented by a linear function, and was the only experiment in which a lower persistence was found with 100 p.p.m. as compared with 50 p.p.m. (Table II). This new set of emulsions behaved otherwise almost identically with the original 1947 set.

TABLE II

Concentration NAA (p.p.m.)	0	5	10	50	100
Persistence (days)	7.83	9.95	12.82	41.02	29.72

Values bracketed together do not differ significantly

One other point of interest emerged from the first experiment comparing NAA and DPOA. Treatments were applied on 8 May 1948 to blossom pedicels; these are slender cylindrical organs and show negligible swelling of the bases, which is a feature of mature fruit stalks. It was observed that in pedicels which persisted for long periods those treated with DPOA had a less 'mature'

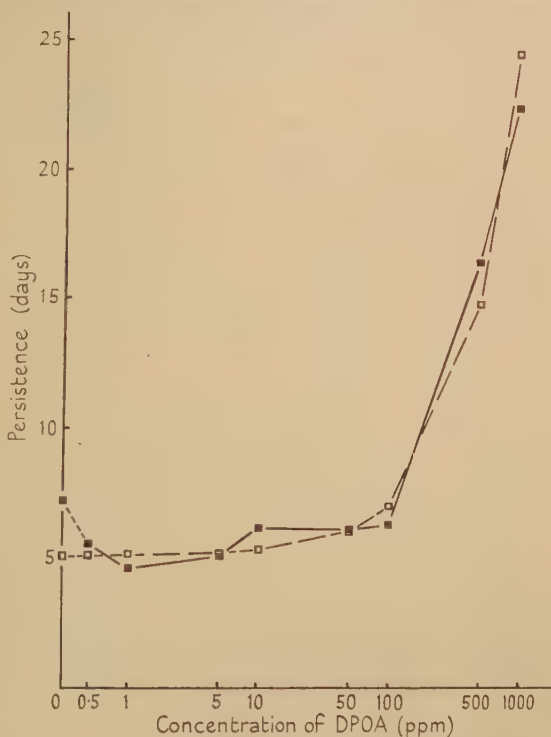


FIG. 3. Relation between concentration of DPOA and persistence of defruited Cox pedicels. Continuous line, observed values; broken line, calculated.

appearance than their NAA counterparts. Measurements showed that the percentage increase in swelling of the base over the uniform middle region was quite different for the two compounds, being 57 per cent. and 33 per cent. for NAA and DPOA respectively.

This 'formative' influence of NAA was observed too late in the season for further comparisons to be made on pedicels, but an experiment on rapidly growing shoots showed the same effect of NAA on increase of diameter of young internodes while DPOA had none.

Rapidly growing Cox vegetative shoots were treated in the following ways: (1) untreated; (2) decapitated, removing the tip down to the first expanded

leaf; (3) decapitated and lanolin emulsion containing 100 p.p.m. NAA applied to the cut surface; (4) as (3), but 100 p.p.m. DPOA applied. Extension of the first internode was curtailed by decapitation, though the second was not affected, and neither growth-substance had much effect on increase in length. Increase in diameter, however, was reduced in both internodes by decapitation, and while NAA had a marked ameliorating influence, DPOA improved

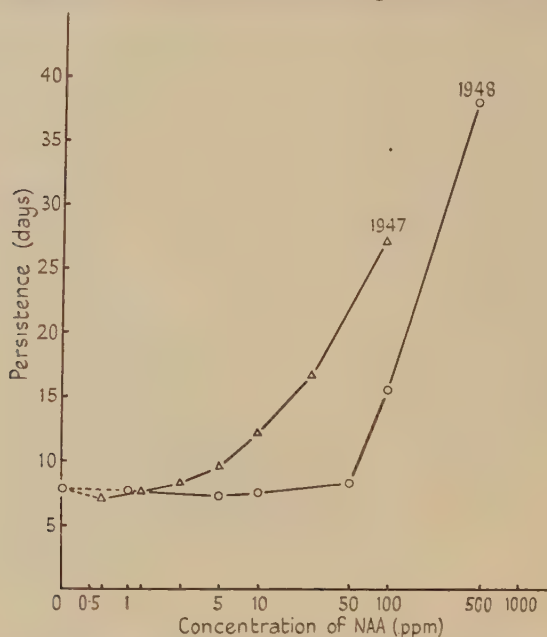


FIG. 4. Relation between concentration of NAA and persistence of defruited Cox pedicels, comparing emulsions made up in 1947 and 1948 in a single experiment in 1948.

the situation only at first, failing in the later period. Initial diameters were very close in all the treatments and little growth occurred after 20 days; the percentage increases in diameter for the first and second internodes are given in Table III after 8 and 20 days from treatment.

TABLE III

Cumulative percentage increases in diameter of the top two internodes of Cox shoots, treated with NAA and DPOA at 100 p.p.m.

Internode		Treatment									
		Untreated				Decapitated		Decapitated + NAA		Decapitated + DPOA	
		1		2		1		2		1	
Days from start		8	34	39	21	27	36	56	33	33	
		20	54	49	35	36	53	67	33	39	

It should be noted that in both pedicels and internodes the increase in diameter was of a normal nature, and not the abnormal swelling reported by Portheim (1941) on bean petioles.

Another contrasting mode of behaviour of NAA and DPOA was illustrated in an experiment using the agar cylinder technique on young (blossom) pedicels of Cox. Here three blossoms were selected on each spur and the flowers were cut from all three; one of the three only was treated. There were five replicates in this experiment, giving five treated and ten adjacent untreated pedicels per treatment.

Table IV shows the results.

TABLE IV
Persistence in days of Cox pedicels

Conc. of growth substance, p.p.m.	NAA		DPOA	
	Treated	Adjacent untreated	Treated	Adjacent untreated
0 (no agar)	—	7.45	—	—
0 (agar only)	6.89	6.95	—	—
1	11.12	7.36	7.76	5.65
10	7.11	6.28	4.47	6.68
100	13.37	8.45	8.83	6.95
1000	22.39 (a)	6.52	27.04* (a)	24.15 (b)

* Including 3 dead pedicels.

(a) Significantly different from controls.

(b) Significantly different from all other untreated pedicels.

The results are unfortunately very variable, but show an aspect in which NAA and DPOA differ in their behaviour; with the former there is no 'leakage' up into the untreated pedicels from the treated, even at the very high concentration of 1,000 p.p.m., while with the latter not only was there considerable delay in abscission of untreated stalks adjacent to those treated with 1,000 p.p.m., but the tips of some of these were blackened and dead.

This raises the question of where the growth-substance must be applied to be effective in inhibiting abscission.

2. Site of application of growth-substance

At least on pedicels or petioles from which the terminal organ has been cut off it would seem that to be effective in preventing abscission the growth-substance must be applied distally to the abscission layer, unless at a very high concentration. With attached organs, however, sufficient may be absorbed in the transpiration stream to return in a basipetal direction and thus bring about inhibition of abscission. This is suggested in the work of Batjer and Thompson (1948), where drops of NAA at 2,400 p.p.m. and sprays at 10 p.p.m. applied to spur leaves delayed fruit fall as effectively as when applied to the stalk. Also branch injections have been effective with NAA and DPOA at 10 p.p.m. (Edgerton and Hoffman, 1948).

Experimental treatments and results are included with others in the next section.

3. Distance of applied growth-substance from the abscission layer

An experiment in 1947 showed that the length of the cut pedicel had a large influence on the effect of NAA on abscission. Lanolin emulsion containing NAA at 10 p.p.m. was applied to the cut surface of pedicels 1.5 cm. long and a half and a quarter this length. It was also applied over a small shallow V-shaped incision made at the constriction zone—the region of the abscission layer—and over one made on the spur 1–2 mm. below the constriction. Similar treatments were made using plain lanolin emulsion. Results are shown in Table V.

TABLE V
Persistence in days of treated Cox pedicels

Site of application	NAA	
	0	10
On tip of full-length pedicel . . .	8.32 (a)	21.93 (b)
„ half- „ „ . . .	8.32 (a)	28.71 (b)
„ quarter- „ „ . . .	8.81 (a)	45.81 (c)
At abscission zone . . .	8.32 (a)	25.64 (b)
On spur below abscission zone . . .	9.14 (a)	9.14 (a)

The letters indicate the three groups into which the treatments fall, treatments within any group not differing significantly from one another, but differing significantly from those in other groups.

It is surprising, in view of the large increase in effect with the shorter pedicel length, that the greatest effect should not occur when application was at the abscission region, and it can only be conjectured that in this treatment part of the effect was dissipated by movement of the growth-substance directly into tissues below the abscission layer through the proximal cut surface of the incision.

More detailed experiments in 1948 separated the effect of pedicel length itself from distance of application of the growth-substance from the abscission layer, and also examined the movement of the growth-substance through undamaged epidermis compared with that through an incision. Results of one experiment are given in Table VI. All pedicels were full length (1.5 cm.) and the side applications were half-way down.

TABLE VI
Persistence in days of Cox pedicels with applications at different sites of NAA and DPOA at three concentrations

Site of application	Control	NAA p.p.m./				DPOA p.p.m.		
		0	10	100	1000	10	100	1000
Cut surface of tip . . .	7.00	7.90	12.33	68.89	7.19	10.61	26.77	
Side . . .	7.58	8.19	18.09	77.89	7.38	7.76	22.89	
Side, over incision . . .	7.58	8.56	28.15	122.00	7.19	13.31	37.39	
Spur, below abscission zone . . .	7.97	7.38	7.79	9.44	7.19	7.19	10.86	
Ditto, over incision . . .	7.58	7.76	8.79	18.62	7.93	7.93	18.54	

These results admit four conclusions. (1) NAA caused significantly greater persistence than DPOA when applied above the abscission zone, but there

was no significant difference between the substances when applied below it. (2) For applications above the abscission zone, 'position' did not interact with 'substance'. Both substances were therefore taken together, and it was found that side applications only gave significantly greater persistence than tip applications if an incision were made. The average values in days were: tip, 15.31; side, 15.67; side with incision, 21.83. It should be remembered that as the growth-substance is applied over the whole cut surface of the tip, application over an incision on the side is most nearly comparable for ease of entry of the substance, so that these results are in line with those of the previous experiment, where a shorter distance to the abscission zone was advantageous. (3) For side applications an incision resulted in significantly greater persistence with concentrations of 100 and 1000 p.p.m., but had no effect at 10 p.p.m. Below the abscission zone, on the other hand, an incision resulted in a significantly greater effect only at the highest concentration. (4) It was only under these last conditions that a growth-substance applied below the abscission zone had any significant effect on persistence at all (i.e. 1,000 p.p.m. over an incision on the spur).

A second experiment examined the effect of pedicel length itself more closely. Here the NAA at a single concentration of 50 p.p.m. was applied to the cut surfaces of the tips of pedicels of full, half, and quarter lengths, and also over incisions made on the side of the pedicel at equivalent 'half' and 'quarter' positions on full-length and at the 'quarter' position on half-length stalks. Thus 'pedicel length' was separated from 'distance of growth-substance application from the abscission zone', and the results, given in Table VII, make it clear that only this latter factor is important.

TABLE VII

Persistence in days of Cox pedicels treated in various positions with lanolin emulsion containing NAA at 50 p.p.m.

Conc. Of NAA	Position of growth-substance application	Distance of growth-substance from abscission zone		
		Full (1.5 cm.)	Half	Quarter
0	On tip	10.64 (a)	12.47 (a)	13.93 (a)
50	On tip	14.89 (a)	52.72 (b)	61.66 (b)
50	On side of full-length pedicel	—	62.23 (b)	54.45 (b)
50	On side of half-length pedicel	—	—	46.88 (b)

The letters indicate two groups into which treatments fall; (a) differ significantly from (b); differences within each group are non-significant. Thus there is no significant effect of growth-substance applied to the tip (which would have been expected from a concentration as high as 50 p.p.m.), nor do the shortest and middle distances differ significantly, though in the experiment described on p. 274 the shortest distance was more effective than the two longer ones.

These results are in opposition to those of some other authors. Portheim (1941) writes: 'It is also interesting to note that Mai (1934) found that the

greater the distance between the pollinium and the basis of *Coleus* petioles the more likely is the petiole to remain attached to the plant.' Portheim himself found the same effect with β -indolylacetic acid applied to debladed petioles of *Phaseolus vulgaris*, while Gardner and Cooper (1943) obtained similar results on *Coleus* petioles of different lengths with compounds of the indole group. With substances of the naphthalene group, however, they found that the greater the length of the petiole, the slower was the initiation of the effect, although when this effect had been brought about, greater length of petiole then also increased it.

This difference between the results presented here and those of the authors cited above, may probably be accounted for by the fact that while my experiments have been conducted on pedicels theirs have been on petioles. An experiment on petioles in progress at the time of writing, though not completed, suggests that there is little effect of petiole length with NAA, though if anything the shorter petioles fall before the longer, in contrast with the pedicel results and in line with those of the authors mentioned. If this indication is borne out by the completed experimental results, the contrast between the effect of length in the pedicels and petioles of apples raises a most interesting question, which will need further investigation.

4. 'Period of reaction' of the pedicel and petiole to applied growth-substance

When the blossom or fruit is cut off its pedicel, or the lamina from its petiole, a train of events is started which leads eventually to abscission, unless a suitable growth-substance application is made. In the absence of an inhibiting agent, however, in these experiments abscission usually takes at least 7 days to be completed, and from observations made on the strength of the abscission layer by pulling off pedicels with a special spring balance, it appears that at least the final stages of weakening of the abscission layer probably take place within 24 hours.

It was thought, therefore, that there must be some critical period in the history of the abscission layer following removal of the terminal organ after which abscission is inevitable, whether or not a growth-substance be applied, and conversely that there must be some period for which the growth-substance has to be present to procure an effect at all.

Experiments to ascertain these periods have not been entirely satisfactory, but some of them are sufficiently suggestive of future lines of work to be included here.

The general method was to remove the terminal organ from its stalk, and either to leave this untreated for a time before applying the growth-substance in lanolin emulsion to the cut surface of the tip, or to apply it immediately for a limited period and then remove it by cutting off the extreme tip of the stalk, carrying the lanolin with it.

Fig. 5 shows the results of two experiments with Cox pedicels, carried out within a few days of each other. It will be seen that the curves for periods without and with NAA are roughly mirror images. A delay in application of

one day seriously reduced the effectiveness of the NAA, but further delays of up to 5 days were not any more deleterious than one. Conversely, applying NAA for a single day has been as effective as for a 5-day period, but all fall short of the effect of leaving the growth-substance on indefinitely.

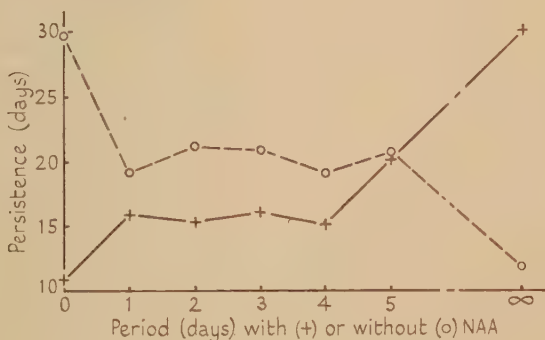


FIG. 5. Effect of delaying application of NAA at 10 p.p.m. for a period after defruiting Cox pedicels (o, broken line), and of applying NAA immediately after defruiting for a limited period (+, continuous line). Separate experiments begun respectively on 1 and 6 September 1947.

This long level middle section of the curves was not expected, and a satisfactory explanation has not yet been arrived at.

An experiment on petioles, however, gave sharper results, as shown by the curves in Fig. 6. Here NAA in lanolin emulsion at 50 p.p.m. was employed and both delayed and limited application periods were included in the same experiment; an additional factor was also introduced. It was felt that in the longer periods without growth-substance, or after the shortest periods with it, unequal drying out of the petiole might occur as compared with those treatments in which the cut surface was protected by lanolin emulsion; parallel treatments were therefore included in which plain lanolin emulsion was applied to the cut surfaces as well as leaving them unprotected. In the half of the experiment concerned with applications of growth-substance for certain periods followed by its removal, the precaution of covering the cut surface with plain emulsion proved unnecessary, there being no significant differences at all between the twelve treatments, all of which were significantly different from the controls. In the half concerned with delayed application, however, there was some effect of protecting the cut surface with plain emulsion before applying the growth-substance. For delays of not more than 2 days lanolin had no significant effect and did not interact significantly with period; the delay of 2 days significantly reduced persistence as compared with no delay. For delays of 4 or 8 days, plain lanolin emulsion applied during the delay period

significantly increased persistence. There was no significant difference between these two periods, but all data from them gave significantly lower persistence than 2 or fewer days delay.

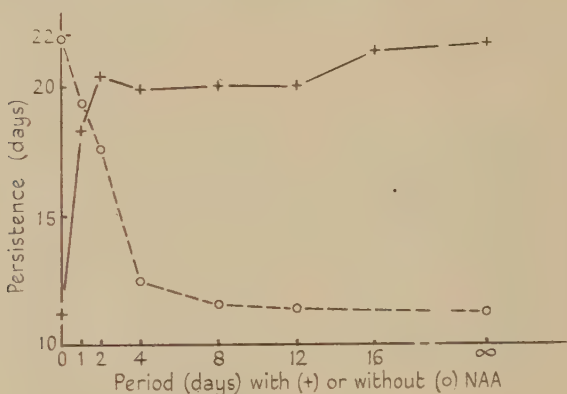


FIG. 6. Effect of delaying application of NAA at 50 p.p.m. for a period after deblading Worcester petioles (o, broken line), and of applying NAA immediately after defruiting for a limited period (+, continuous line).

Table VIII summarizes these findings, while the graphs in Fig. 6 show the mean values for the 'protected' and 'unprotected' treatments.

TABLE VIII

Persistence in days of petioles treated with NAA at 50 p.p.m. after a period without growth-substance

Period of delay (days)				
0	1	2	4 or 8 with protective lanolin	4 or 8 without protective lanolin
21.88	19.28	17.34	12.91	11.14

Values bracketed together do not differ significantly

It is clear from this experiment on debladed petioles that the critical period of reaction of the abscission layer to the presence or absence of growth-substance is within the first 48, or possibly 24, hours after removal of the lamina, although completion of the process does not occur until some 11 days after deblading.

Further work is necessary to establish whether the pedicel behaves in a similar manner, or whether, as suggested in the previous experiments, there are two critical periods, one for initiation of the process and one for its completion, either of which may be inhibited by appropriate applications of NAA.

DISCUSSION

The working hypothesis adopted in this research to account for the hormonal control of abscission is as follows: an organ capable of abscission tends to separate from the plant, but the process of abscission is inhibited as long as the supply of a natural or synthetic growth-substance is maintained above a certain level. This growth-substance must be presented at the abscission zone from a distal position.

The experiments described above fit this general hypothesis and allow some extension in certain details: (*a*) for the growth-substances NAA and DPOA the inhibition of abscission usually bears a direct linear relation to the concentration; (*b*) on pedicels, at a given concentration, these substances are more effective the nearer they are applied to the abscission layer (provided the application is distal to it), though on petioles this may be reversed; (*c*) there is probably a critical period shortly after deprivation of the natural hormone supply in which abscission is initiated, and to be effective, synthetic growth-substance must reach the abscission zone within this period.

One important factor has been investigated without so far reaching a conclusion, and that is whether it is the *amount* of growth-substance reaching the abscission zone, or the *concentration* at the zone, which is of paramount importance in producing an inhibiting effect on the abscission process. Experiments to date have given results which can be explained wholly in terms of either amount or concentration, without any indication of which is the more likely to give a unique explanation.

Furthermore, it must be emphasized that this paper has only dealt with factors affecting the inhibition of abscission, and no attempt will be made here to suggest a causative mechanism, beyond indicating that it is probably an agent in the plant body as a whole, and not one in the organ to be abscised, since it is removal of this which leads to activation of the abscission process. An indication that the activator is of the nature of ethylene will be dealt with later in these studies.

Nor do the experiments described above give any clue as to the means by which inhibition of the abscission process is achieved by these growth-substances, and indeed it is difficult to suggest a mechanism of inhibition until one of activation has been ascertained.

SUMMARY

1. The general method is described for experimenting on apple pedicels or petioles from which the terminal organ has been removed.
2. Two quantitative techniques for applying growth-substances are given in detail, one employing lanolin emulsion as the carrier, the other agar gel.
3. By these methods certain factors affecting the inhibition of abscission by the growth-substances α -naphthylacetic acid (NAA) and 2:4-dichlorophenoxyacetic acid (DPOA) have been investigated.

A relation between concentration of the growth-substance and persistence

of the pedicel was established which was usually linear. In an experiment with NAA, for instance, the relation was represented by—

Persistence in days = $6.52 + 0.0127 \times \text{concentration in p.p.m.}$

4. The acid and the sodium salt of both NAA and DPOA gave similar results when applied in lanolin emulsion.

5. On both young pedicels and young shoot internodes NAA was found to exert a 'formative' influence resulting in increase in diameter which was not exhibited by DPOA to the same extent.

6. At least for concentrations of either growth-substance up to 100 p.p.m., these were effective only when applied distally to the abscission zone. At 1,000 p.p.m. there was an effect of application on the proximal side of the abscission region, but it was small compared with the effect of the same concentration applied distally.

7. The length of the path of the growth-substance from the point of application to the abscission zone affected the degree of inhibition inversely, the longer the path the less the effect of a given concentration in delaying abscission. The contrast between these findings for pedicels, and those of other authors for petioles, is briefly discussed.

8. A critical period occurs shortly after removing the leaf-blade from its petiole during which the abscission process is initiated, and to be effective in inhibiting this process growth-substance must reach the abscission zone within this period. It is not at present established whether the pedicel shows a similar mode of behaviour.

9. A working hypothesis is given to account for the control of abscission by natural hormones, and extensions are described to embrace the results of experiments with synthetic growth-substances.

The writer wishes to thank Mr. J. Taylor, of the Statistics Section, East Malling Research Station, for the considerable work entailed in analysing many of these experiments.

LITERATURE CITED

- BARLOW, H. W. B. (1938). Report on year 1937-8, to Botany Dept., Edinburgh University. *Unpublished*.
- (1948). Intensive experimental methods in abscission studies. *Rep. E. Malling Res. Sta. for 1947*, 121-4.
- BARNELL, E. (1939). Studies in tropical fruits. V. Some anatomical aspects of fruit-fall in two tropical arboreal plants. *Ann. Bot.*, N.S., **3**, 77-89.
- BATJER, L. P., and MARTIN, P. C. (1945). New materials for delaying fruit abscission in apples. *Science*, **101**, 363.
- , and THOMPSON, A. H. (1948). The transmission of effect of naphthalene acetic acid on apple drop as determined by localized applications. *Proc. Amer. Soc. Hort. Sci.* **51**, 77-80.
- BEAL, J. M., and WHITING, A. G. (1945). Effect of indoleacetic acid in inhibiting stem abscission in *Mirabilis jalapa*. *Bot. Gaz.* **106**, 420-31.
- EDGERTON, L. J., and HOFFMAN, M. B. (1948). Tests with 2, 4-dichlorophenoxyacetic acid for delaying fruit drop of McIntosh. *Proc. Amer. Soc. Hort. Sci.* **51**, 67-70.
- GARDNER, F. E., and COOPER, W. C. (1943). Effectiveness of growth substances in delaying abscission of *Coleus* petioles. *Bot. Gaz.* **105**, 80-9.

- GARDNER, F. E., and MARTH, P. C. (1939). Effectiveness of several growth substances on parthenocarpy in Holly. *Ibid.*, **101**, 226-9.
- — —, and BATJER, L. P. (1939). Spraying with plant growth substances to prevent apple fruit dropping. *Science* **90**, 208-9.
- — — (1939a). Spraying with plant growth substances for control of the preharvest drop of apples. *Proc. Amer. Soc. Hort. Sci.* **37**, 415-28.
- HARLEY, C. P., MOON, H. H., and REGEIMBAL, L. O. (1947). Further studies on sprays containing 2, 4-dichlorophenoxyacetic acid, and some related compounds, for reducing harvest drop of Apple. *Ibid.* **50**, 38-44.
- HEINICKE, A. J. (1919). Concerning the shedding of flowers and fruit and other abscission phenomena in apples and pears. *Ibid.* **17**, 76-84.
- LAIBACH, F. (1933). Versuche mit Wuchsstoffpaste. *Ber. d. d. bot. Ges.* **51**, 386-92.
- LA RUE, C. D. (1936). The effect of auxin on abscission of petioles. *Proc. Nat. Acad. Sci.* **22**, 255-9.
- LLOYD, F. E. (1914). Injury and abscission in *Impatiens sultani*. *Sixth report of the Quebec Society for the protection of plants*, 72-9.
- (1916). Abscission in *Mirabilis jalapa*. *Bot. Gaz.* **61**, 213-30.
- (1927). Abscission in general and with special reference to the curtailment of fruitage in *Gossypium*. *Mem. New York Hort. Soc.* **3**, 195-207.
- MAI, G. (1934). Korrelationsuntersuchungen an entspreiteten Blattstielen mittels lebender Orchideenpollinien als Wuchsstoffquelle. *Jahrb. f. wiss. Bot.* **79**, 681.
- MARTH, P. C., BATJER, L. P., and MOON, H. H. (1945). Relative effectiveness of dusts, sprays and aerosols of naphthaleneacetic acid on harvest drop of apples. *Proc. Amer. Soc. Hort. Sci.* **46**, 109-12.
- MCCOWN, M. (1939). Abscission of flowers and fruits of the apple. *Abstct. in Proc. Amer. Soc. Hort. Sci.* **36**, 320.
- (1943). Anatomical and chemical aspects of abscission of fruits of the apple. *Bot. Gaz.* **105**, 212-20.
- MACDANIELS, L. H. (1936). Some anatomical aspects of apple flower and fruit abscission. *Proc. Amer. Soc. Hort. Sci.* **34**, 122-9.
- MYERS, R. M. (1940). Effect of growth substances on the absciss layer in leaves of *Coleus*. *Bot. Gaz.* **102**, 323-38.
- PORTEIM, L. (1941). Further studies on the action of heteroauxin on *Phaseolus vulgaris*. *Ann. Bot., N.S.*, **5**, 35-46.
- SAMPSON, H. C. (1918). Chemical changes accompanying abscission in *Coleus blumei*. *Bot. Gaz.* **66**, 32-53.
- SAMUEL, G. (1927). On the shot-hole disease caused by *Clasterosporium carpophilum* and on the 'Shot-hole' effect. *Ann. Bot.* **41**, 375-404.
- SCOTT, F. M., SCHROEDER, M. R., and TURRELL, F. M. (1948). Development, cell shape, suberization of internal surface, and abscission in the leaf of the Valencia orange, *Citrus sinensis*. *Bot. Gaz.* **109**, 381-411.
- ULRICH, R. (1944). La Chute des Fruits. *La Revue Scientifique* **82**, 25-34.
- VYVYAN, M. C. (1946). Fruit fall and its control by synthetic growth substances. *Tech. Commun. Imp. Bur. Hort. and Plant. Crops* **18**.
- — —, and BARLOW, H. W. B. (1947). Use of sprays to control fruit-drop, especially that of young fruit of Cox's Orange Pippin. *Rep. E. Malling Res. Sta. for 1946*, 83-7.
- WHITING, A. G., and MURRAY, M. A. (1948). Abscission and other responses induced by 2, 3, 5-triiodobenzoic acid in bean plants. *Bot. Gaz.* **109**, 447-73.
- WINKLEPLECK, R. L., and MCCLINTOCK, J. A. (1941). Lanolin emulsions as carriers of growth substances. *Proc. Amer. Soc. Hort. Sci.* **38**, 94-6.

The Absorption of Ions by Excised Root Systems

I. APPARATUS AND PRELIMINARY EXPERIMENTS

E. C. HUMPHRIES

Rothamsted Experimental Station, Harpenden, Herts.

INTRODUCTION

THE work to be described in this paper originated in an attempt to develop a method of measuring the physiological activity of roots which could be applied to roots growing in the soil under different growth conditions such as of nutrition and aeration. One of the most important properties of roots is their ability to absorb nutrients from dilute soil solutions and to accumulate them within the plant. With this in mind some preliminary experiments were performed in which the absorption capacity of whole excised root systems, from plants grown under different conditions of nutrition, was measured when the roots were placed under standard conditions of complete nutrient supply, constant temperature, and adequate aeration. These preliminary experiments had as their object an answer to the question, Do excised roots of plants grown in conditions of deficiency of a particular cation or anion absorb that ion when they are placed in a complete culture solution? Previous work has shown that this probably is the case, but the question has never been specifically investigated. The important implications in the study of nutrient uptake by crop plants need hardly be stressed. Most previous investigations of nutrient uptake by roots were concerned with the entry of one particular ion, or, at the most, the absorption of a cation and its associated anion. An exception is the work of Woodford and Gregory (1948), who studied the simultaneous uptake of NO_3^- , H_2PO_4^- , and K^+ by young, intact, barley plants. In the present experiments the absorption of potassium, nitrogen, and phosphorus by the same root system has also been studied, but in all cases excised roots have been used. It was originally planned to use only the growing root-tip (a length of about 2 cm. from the tip) since this has been shown by various investigators (Prevot and Steward, 1936; Gregory and Woodford, 1939; Steward, Prevot, and Harrison, 1942; Machlis, 1944) to be the region of most rapid absorption. It was found in practice that the large amount of plant material needed, and the difficulty of completing sampling in a reasonable time, precluded this procedure and the only practicable alternative was to use the whole root system of a young plant as the unit. This introduces the difficulty that the system then contains cells of widely different absorbing power, and it is possible that some cells in the system may be losing nutrients at the same time that others are absorbing them. The results from a single experiment using root tips only will be quoted.

Some experiments were also performed with root systems of plants grown in soil. The object of this was first to see whether roots could be recovered from soil without undue damage, and if so, whether the excised root systems of these behaved similarly to those grown in an aerated water culture. If this were the case it would afford a possible approach to the problem of diagnosis of nutrient deficiency.

DESCRIPTION OF CULTURE TECHNIQUE

The plants for these experiments were grown in tanks¹ of 2½-gal. capacity made from asphalt and asbestos. These are preferable to metal tanks, for they are not corrodible and there is no evidence, after more than 2 years' use, that the material is in any way toxic to plants, or that it exchanges ions with the culture solution. The tanks, which are slightly wider at the top than at the bottom, are filled with 'desalted' water (see below) up to the ledge which normally indicates the water level when the tank is used for its intended purpose. On this ledge is fitted a wooden grid (17¼ in. × 7½ in. × ⅜ in.) thoroughly impregnated with paraffin wax and then painted with bitumastic paint. Four longitudinal slits (16½ in. × ¼ in.) are cut through the grid, slightly recessed by a bevelled groove and extending almost the whole length of the grid. Over these slits are placed pieces of waxed netting, approximately 1 in. wide with meshes about ⅛ in. square. The length of the waxed strips varies according to the number of samples it is desired to take from each tank. Seeds were germinated in glass germinators on filter-paper until the roots are about 1 cm. long and then placed on the waxed strips with the roots projecting through the holes. The number of seeds per strip depended on the plant species. For example, usually 72 barley, 24 bean, or 36 pea plants were grown in each tank. When the plants were well established (2 or 3 days) the appropriate culture solutions in concentrated form were measured into each tank. The basic (or complete solution) was usually half-strength Hoagland solution (Hoagland and Broyer, 1936) having the following composition:

Ca (NO ₃) ₂	.	.	.	0.0025 M
KNO ₃	.	.	.	0.0025 M
MgSO ₄	.	.	.	0.001 M
KH ₂ PO ₄	.	.	.	0.0005 M

In addition 5 ml. of 0.5 per cent. ferrous tartrate and 5 ml. of Hoagland's A4 solution were supplied to each tank. The water used was prepared by the Siemens process of electro-osmosis and was very low in salts, but still contained traces of calcium. This water may be conveniently referred to as 'desalted water'.

In some experiments the culture solutions were renewed during the growth of the plants; in others the solutions were allowed to become progressively weaker by absorption. No forced aeration of the culture solution

¹ Actually water-closet cisterns manufactured by Pritchett and Gold and E.P.S. Ltd., Dagenham Dock, Essex.

was employed, but apparently there was adequate exchange of oxygen between the air and solution through the large water surface for normal growth. Oxygen renewal also took place when the solution level was restored by the addition of water.

Sampling was conveniently carried out by lifting strips of netting from the grooves and cutting off the roots in distilled water. The strips in each tank were selected at random according to a pre-arranged plan. Each sample was drawn from a number of tanks.

DESCRIPTION OF APPARATUS

The apparatus used consisted of a large thermostat containing brown glass bottles of 500-ml. capacity in which the roots were placed and subjected to a constantly renewed culture solution and a stream of air. The apparatus is shown diagrammatically in Fig. 1. A pressure maintained in the large aspirator A pumped culture solution through a length of capillary B into the bottom of a 750-ml. filter flask C where the solution attained the temperature of the bath. A stream of air was also passed into the top of the filter flask, through a small length of capillary D, by means of a pump. The air stream passed into the bottle E, containing the roots, through a sintered-glass disk (porosity o) and at the same time carried over culture solution which was flowing into the filter flask. Both air and solution were thus distributed through the sintered disk into the root vessel. Air and liquid passed out of the root vessel through the glass tube F and went to waste. The head of liquid, measured on the manometer G, was maintained constant by means of the pressure bottle H, which was connected by means of a glass tube to a small bottle J, containing mercury. Air was pumped into H through the non-return valve K by means of a small 'Dymax' air pump and mercury was forced up the tube L to make contact with an adjustable contact M. Another contact, N, consisting of a steel knitting needle dipped under the surface of the mercury in the bottle. When electrical contact at M occurred the pump motor was switched off through a relay. As soon as the pressure fell and contact was broken at M the motor was switched on again and the pressure in H and hence the pressure over the solution in the aspirator was maintained. This arrangement gave a head with a variation of less than 1 cm. of solution. Each root vessel was fed with solution by a separate line of transparent plastic tubing (which enables air bubbles to be readily detected and is more easily manipulated than glass). A length of capillary tubing B was inserted into each line. This ensured that the rate of flow to each of the bottles was approximately the same. Similarly, a small length of capillary tubing D was inserted in each of the air lines to distribute the air more evenly between the bottles. The actual rate of flow of air was controlled by the depth to which the tube P, attached to the end of the air line, dipped under a column of water held in a wide glass tube Q. By increasing this depth the rate of flow through the bottles was increased so long as air was still escaping through the end of the tube. The thermostat tank of 200-gal. capacity was heated by means of a $1\frac{1}{4}$ -kw. electric heater. The temperature of the

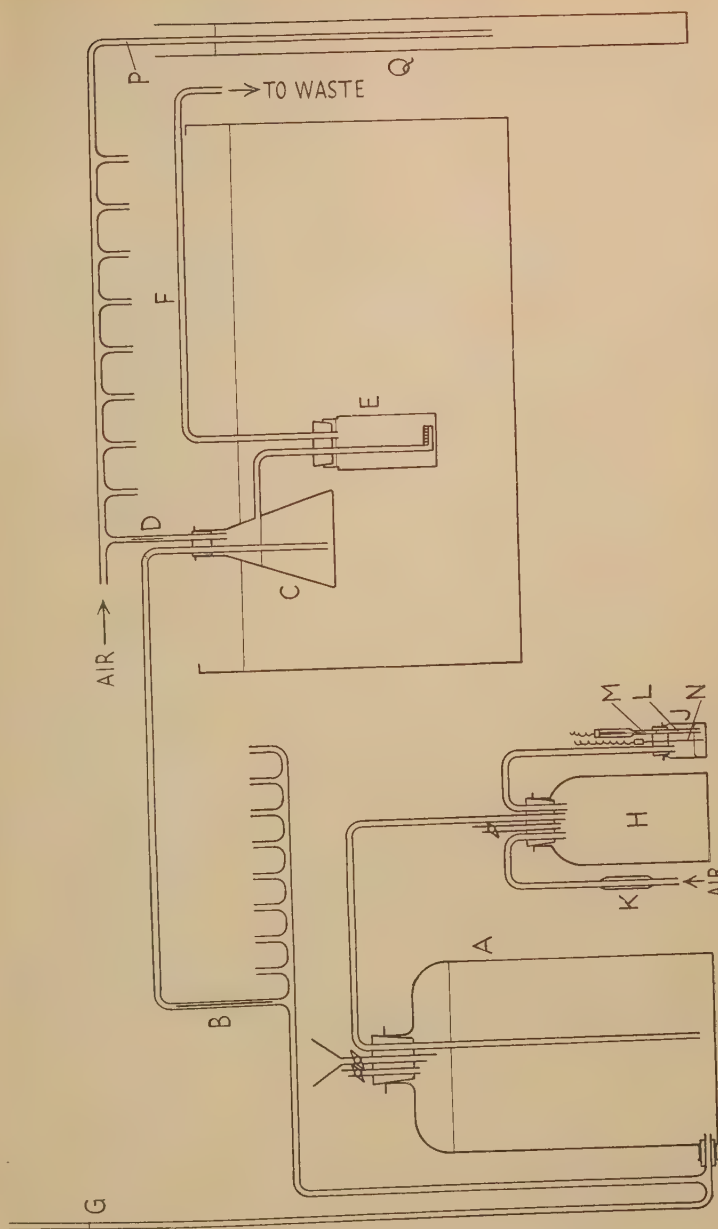


FIG. 1. Diagram of absorption apparatus. For explanation see text.

thermostat was 25° C. in all experiments except No. II, where it was 30° C. In all the experiments to be described the culture solution used in the absorption apparatus was *full-strength* Hoagland solution or twice the strength shown on p. 283. The experimental arrangement was designed to maintain the nutrient content, pH, and temperature of the solution surrounding the roots as nearly constant as possible throughout the absorption period and to provide optimal conditions for absorption.

The use of a culture solution such as Hoagland's, containing uni- and bi-valent ions, should tend also to give optimal conditions for the absorption of ions. Mattson, Eriksson, Vahtras, and Williams (1949), from theoretical considerations of the Donnan equilibrium, showed that the presence of cations should have an effect on the uptake of anions such as phosphate and nitrate. The effect of bivalent cations should be greater than the effect of monovalent cations, and it was demonstrated experimentally that the presence of potassium and calcium increased the uptake of phosphate by excised barley roots. Viets (1942, 1944) also showed that the absorption of potassium and bromide ions by excised barley roots was increased in the presence of calcium bromide, and he demonstrated that other ions such as Mg, Sr, Ba, and Al increased potassium accumulation.

SAMPLING TECHNIQUE

The root systems were cut off at the junction of stem and root, washed in distilled water, and centrifuged in a perforated cylindrical metal basket of 7 in. diameter at 2,000 revs. per min. for exactly 2 minutes. It was found that centrifuging for a longer period of time did not remove any more water from the surface of the roots and the procedure gave reproducible values for the water content of the fresh material. Immediately after centrifuging the roots were placed in a tin and weighed. They were then placed in the absorption apparatus for a prescribed time, usually 20 to 24 hours. At the end of the absorption period they were taken out of the apparatus, washed in distilled water, centrifuged, weighed, and dried. A duplicate sample of roots was centrifuged, weighed, and dried to provide data on the initial content.

There was invariably a change in fresh weight of the roots during the absorption period and all results presented in the tables have been expressed in terms of the initial fresh weight. The initial sample which was oven-dried without further treatment will be referred to as sample *A*, while the sample which was weighed fresh and subsequently subjected to an absorption treatment, constituted sample *B*. This sample consequently had two fresh weights, initial and final. The percentage change in mineral content referred to in the tables is the increase or decrease as percentage of the initial content. In all cases the 5 per cent. level of probability has been used as the criterion of statistical significance.

ANALYTICAL METHODS

Potassium was determined in the earlier experiments by the method of Piper (1942), but later the silver-potassium cobaltinitrite method of Ismail

and Harwood (1937) was preferred. Nitrogen was determined by the micro-kjeldahl procedure of Markham (1942) and modified to include nitrates by a preliminary treatment of the dried material with salicylic acid (Doneen, 1932). Phosphorus was determined colorimetrically by stannous chloride reduction.

EXPERIMENTAL RESULTS

Experiment I

Seedlings of broad bean (*Vicia sativa*) were germinated and grown in tanks (24 per tank) containing either culture solution (half-strength Hoagland solution) or distilled water only. The plants were sampled after 24 days' growth. 2 cm. apical portions of root tip were removed from lateral roots and placed in ice-cold distilled water to reduce metabolism to a minimum. When the two sets of plants had been sampled, each sample was divided into two and both lots were centrifuged and weighed. One half was dried at 100° C. and the other placed in *full-strength* Hoagland solution in the absorption apparatus for 21 hours. The results of the analyses are shown in Table I.

The material available was sufficient only for a single replication so that no estimate of error is possible. There was an increase in the phosphorus and potassium contents of 60 per cent. in the roots of the plants grown in distilled water. The increase in nitrogen content was small (5.7 per cent. of the initial value). The initial content of nitrogen of these roots, however, was quite high, in fact of the same order as that of the roots grown in culture solution. The contents of the three elements in the root tips of the plants grown in culture solution showed little change after treatment except for a 10 per cent. decrease in the nitrogen content. It will be noticed from Table I that there was a slight loss in fresh weight of the root tips of both treatments after the period of absorption. This was commonly observed in experiments of this kind with plants grown in culture solutions. It appears to be due in part to loss of water and in part to loss of dry matter (carbohydrates, &c.) by respiration, as well as, in some cases, loss of ions to the external solution. It will be noticed that the dry matter expressed as a percentage of the initial fresh weight is lower in the treated samples than in the controls.

This preliminary experiment showed that roots of plants with an induced deficiency (grown in distilled water) were capable of absorbing phosphorus and potassium and to a lesser extent nitrogen from a complete aerated culture solution.

Experiment II

Dwarf bean plants (*Phaseolus vulgaris* var. 'Canadian Wonder') were grown in the following solutions: (a) complete nutrient (half-strength Hoagland); (b) as (a) but lacking nitrogen (the chlorides of calcium and potassium in equivalent amount replacing the corresponding nitrates); (c) as (a) but lacking phosphorus (potassium chloride replacing potassium phosphate); (d) as (a) but

TABLE I

The dry matter, nitrogen, phosphorus, and potassium contents as percentage of initial fresh weight of samples of Vicia faba root-tips, grown in culture solution or in distilled water, determined on sample A before treatment and on sample B after treatment with aerated complete culture solution

[illegible]

TABLE II

The dry matter, nitrogen, phosphorus, and potassium contents as percentage of initial fresh weight of samples of Phaseolus (dwarf bean) roots, grown in complete or deficient culture solutions, determined on sample A before treatment and on sample B after treatment with aerated complete culture solution

Cultural treatment	Fresh weight (g.) per 18 roots			% initial fresh weight						% change					
	A	B		Change	Dry weight		N		P		K	N	P	K	
		Initial	Final		A	B	A	B	A	B					
Complete	41.21*	34.17*	0.14	7.58	6.77	0.286	0.276	0.167	0.172	0.474	0.396	—	3.5	3.0	16.5
—K	29.65	27.70	0.15	7.23	6.65	0.302	0.303	0.129	0.140	0.064	0.168	0	0	8.5	+462.5
—N	35.12	36.92	0.39	7.26	6.80	0.160	0.177	0.172	0.183	0.422	0.419	+10.6	+10.6	6.4	—0.7
—P	46.63	49.55	+0.43	7.00	6.21	0.290	0.252	0.025	0.073	0.466	0.390	13.1	13.1	+192.0	—16.3
S.E.	—	—	0.11†	—	—	0.0065†	—	0.0050†	—	0.0085†	—	—	—	—	—

* Owing to an error these samples did not consist of exactly 18 plants.

† 4 D.F.; $t = 2.776$.

17 D.F.; $t = 2.365$.

lacking potassium (sodium salts replacing the potassium salts). The tanks were set out in randomized blocks. The solutions were renewed three times during the growth period of 24 days. Duplicate samples of roots for each treatment (18 roots per sample) were taken according to the usual procedure. The temperature of the thermostat in this experiment was 30°C. The analyses of the roots before and after treatment are shown in Table II.

There was no change in the nitrogen content of the complete, —K, or —N treatments; the increase in nitrogen content of the roots lacking in N just failed to be significant. There was, however, a decrease in the nitrogen content of the —P roots. Only the roots of the —P treatment showed a significant increase in phosphorus content and this was large (192 per cent.). Both the complete and —P treatments showed a significant loss of potassium (approx. 16 per cent.) while the —K treatment showed a considerable gain. There was no change in potassium content of the —N treated plants. It will be noticed that there was a loss of fresh weight in all samples after treatment except in the case of the —P treatments where both samples *gained* in fresh weight. In all treatments there was a loss of dry weight in the final samples compared with initial. Thus in the case of the —P treatment there was an overall gain in fresh weight of the sample during absorption in spite of a loss of dry matter. It will be noted from the data of Table II that the plants grown in the solutions lacking phosphorus had the largest root system—a well-known phenomenon of phosphorus deficiency (cf. Williams, 1948).

Experiment III

In a preliminary experiment with barley some plants were grown in complete nutrient solution (half-strength Hoagland) and others in Harpenden tap-water (low in N, P, and K, and high in Ca). The plants were sampled after 32 days' and again after 39 days' growth. The solutions were changed three times before the first sample was taken and once again before taking the final sample. The plants grown in tap-water showed purple veining at the base of the stems, suggesting acute phosphorus deficiency. The roots were sampled as before and kept at 25°C. for 24 hours. The results are shown in Table III.

There was a loss of nitrogen from the roots of plants grown in full nutrient on both occasions, while the loss from the roots grown in tap-water was not significant. There was no change in the phosphorus content of the full nutrient treatment on either occasion and a considerable gain by the roots grown in tap-water (180 per cent.). Potassium was lost by the roots grown in complete culture on both sampling occasions, but the roots grown in tap-water absorbed potassium on the first occasion. The increase in potassium on the second occasion was not significant. Loss in fresh weight during treatment occurred in both the roots grown in culture solution and in tap-water. The loss of fresh weight on the second sampling occasion was greater than on the first. There was also a decrease in dry weight as percentage of fresh weight in all the samples after absorption treatment.

TABLE III

The dry matter, nitrogen, phosphorus, and potassium contents as percentage of initial fresh weight of barley roots, grown in culture solution or in tap-water, determined on sample A before treatment and on sample B after treatment with aerated complete culture solution

Treatment	Fresh weight (g.)			% initial fresh weight						% change		
	A	B		Dry weight		N		P		K		K
		Initial	Final	Change	Change	A	B	A	B	A	B	
Culture solution:												
32 days	26.88	28.93	27.77	-1.16	5.49	0.252	0.174	0.089	0.086	0.326	0.280	-31.0
39 days	34.71	35.90	33.68	2.22	5.73	0.275	0.242	0.081	0.073	0.309	0.241	12.0
Tap-water:												
32 days	16.49	15.21	14.74	-0.47	7.02	0.184	0.164	0.012	0.034	0.049	0.094	10.9
39 days	21.15	20.24	18.93	1.31	7.98	0.187	0.179	0.014	0.038	0.062	0.064	4.3
S.E.	—	—	—	0.197*	—	0.0055†	—	0.0044†	—	0.0075†	—	—

* 4 D.F.; $t = 2.776$.† 7 D.F.; $t = 2.365$.

Experiment IV

The primary object of this experiment was to see if carbohydrate supplied to the flowing culture solution, to which the excised roots are exposed, would affect the uptake or loss of ions from roots. The experiment was commenced about the middle of October, when the carbohydrate content of barley plants is likely to be low. The plants were grown in tap-water + 3.0 g. potassium nitrate per tank, to provide phosphorus-deficient plants. The plants were sampled after 28 days' growth and after 49 days' growth. Three changes of solution were made during the growth period, all before the first sampling. Samples of excised roots were treated in the usual way with culture solution and in addition samples were treated with the same strength culture solution containing 2 per cent. sucrose. The duration of the experiment was 24 hours. Some bacterial contamination occurred in the sucrose series and the usual time of 2 minutes for centrifuging was insufficient to remove the surplus water from the roots. The fresh weights were obtained in these cases after a total time of 7 minutes' centrifuging. The results obtained are shown in Table IV.

At the first sampling the roots treated with sucrose showed a large increase in nitrogen content (70 per cent.), but at the second sampling the changes were small and not significant. The increase in phosphorus content of the sucrose-treated roots was large at both sampling times. There was no significant change in the potassium content on either occasion. These results are quite different from those obtained in the absence of sucrose. The 25 per cent. loss of nitrogen at both sampling times was barely significant. The increase in phosphorus was large, but only about half of the increase obtained in the presence of sucrose; the increase in the presence of sucrose was significantly higher than that obtained in the absence of sucrose. There were significant losses of potassium in the absence of sucrose.

These results suggest that the presence of extra sucrose not only prevented loss of potassium but also increased the uptake of phosphorus and nitrogen. The experiment is open to the criticism that bacterial contamination present in the sucrose series may have influenced the result. Any uptake by bacterial cells would subsequently be ascribed to the roots if the bacteria adhered to the roots. This possibility could only be tested by using roots from plants grown in sterile conditions.

The samples treated with sucrose increased in fresh weight on the first sampling occasion and also increased in dry weight percentage of initial weight. On the second occasion, however, there was a decrease in fresh weight and no change in dry weight. This suggests that the loss of respiratory carbohydrate was at least made good by the sucrose absorbed. The samples treated with culture solution, without added sucrose, behaved in conformity with previous experiments and showed a loss in fresh weight and dry weight after the absorption period.

TABLE IV

The dry matter, nitrogen, phosphorus, and potassium content as percentage of initial fresh-weight of barley roots grown in tap-water and potassium nitrate, determined on sample A before treatment and on sample B after treatment with aerated complete culture solution in the presence and absence of sucrose

Treatment during absorption	Fresh weight (g.)			% initial fresh weight				% change			
	A	B		Dry weight		N		P		K	
		Initial	Final	A	B	A	B	A	B	A	B
Sucrose:			Change								
28 days	18.70	19.88	22.06*	5.25	6.68	0.186	0.316	0.011	0.057	0.322	0.368
49 days	24.32	24.70	23.26*	7.13	7.11	0.223	0.246	0.010	0.045	0.334	0.359
No sucrose:											
28 days	16.60	17.17	15.65	5.21	4.39	0.180	0.135	0.011	0.028	0.327	0.194
49 days	22.39	22.36	20.57	6.84	5.91	0.224	0.169	0.011	0.037	0.326	0.214
S.E.	—	—	—	—	—	0.0088†	—	0.0009‡	—	0.0122‡	—

* Roots centrifuged for 7 minutes.

† 4 D.F.; $t = 2.776$.‡ 7 D.F.; $t = 2.365$.

Experiment V

This experiment was included to see whether it would be possible to use roots from plants grown in soil as experimental material. A plot of neglected allotment was selected on which there was abundant growth of two weed species, *Lamium purpureum* and *Veronica persica*, in close association. There were practically no other plant species present. The time of sampling was mid-April and both species were flowering freely and fruit formation was beginning in *V. persica*. The plants were dug with a garden fork, as much soil as possible being removed by gentle shaking, and the roots were put in buckets containing ice and water. In the laboratory the two species were separated and soil removed by washing under a gentle stream of water and then the roots were severed and given a final wash in distilled water. Finally they were centrifuged, weighed, and treated in the usual manner at a temperature of 25° C. for 24 hours. The changes during treatment are shown in Table V.

In both species there was a big gain in nitrogen, but no change in the phosphorus or potassium contents. This suggests that the plants were growing in a soil deficient in available nitrogen. Evidently the roots had not lost their capacity for absorption, and it may be concluded that they did not sustain appreciable damage during excavation and washing. A development of the method may be possible in the diagnosis of nutrient deficiencies.

The loss in fresh weight of the roots after treatment of the two species was only slight—just over 1 per cent., and smaller than found with plants (although not of these species) grown in culture solutions.

Experiment VI

A similar experiment to the last was performed with groundsel (*Senecio vulgaris*) dug from a different plot later in the year (end of June). The results of the analyses are shown in Table VI.

The increase in nitrogen was not quite significant, and there was no change in potassium, but there was an appreciable increase in phosphorus (41 per cent.). Thus the results of this experiment are in contrast to the previous one where there was a large increase in nitrogen and no change in phosphorus. Again, in this experiment there was no evidence of damage to the root-cells or loss of capacity for absorption. In both final samples the percentage increase in fresh weight after treatment was about 10 per cent., suggesting that water had also been absorbed, and this may be an indication of a water deficit in the root cells. There was also an increase in dry weight as percentage of initial fresh weight, but this was entirely due to one of the final replicates.

DISCUSSION

As Hoagland and Broyer (1936) point out, the use of excised roots for the study of nutrient absorption eliminates the complications of root-shoot relationships, but at the same time brings difficulties of interpretation. The ultimate aim is to relate information obtained by this technique to the processes which take place in the intact plant.

TABLE V

The dry matter, nitrogen, phosphorus, and potassium contents as percentage of initial fresh weight of roots of Lamium purpureum and Veronica persica, grown in soil, determined on sample A before treatment and on sample B after treatment with aerated complete culture solution

	Fresh weight (g.)			% initial fresh weight						% change		
	A		B	Dry weight		N		P		K		
	Initial	Final	Change	A	B	A	B	A	B	A	B	
<i>Lamium purpureum</i>	19.29	21.10	20.80	0.30	11.1	10.0	0.119	0.184	0.060	0.053	0.451	+54.6
<i>Veronica persica</i>	9.43	10.27	10.15	-0.12	10.9	9.6	0.119	0.172	0.066	0.060	0.333	+44.5
S.E. { <i>L. purpureum</i>	.	.	.	.	.	.	0.0026*		0.00016*		0.037*	—
{ <i>V. persica</i>	.	.	.	.	.	.	0.0021*		0.0036*		0.028*	—

* 2 D.F.; $t = 4.303$.

TABLE VI

The dry matter, nitrogen, phosphorus, and potassium contents of roots of Senecio vulgaris, grown in soil, determined on sample A before treatment and on sample B after treatment with aerated complete culture solution

	Fresh weight (g.)			% initial fresh weight						% change		
	A		B	Dry weight		N		P		K		
	Initial	Final	Change	A	B	A	B	A	B	A	B	
13.79	13.51	14.88	+1.37	7.07	7.44	0.215	0.231	0.036	0.051	0.432	0.449	+7.4
S.E.				0.0046*		0.0046*		0.0020*		0.016*		—

* 2 D.F.; $t = 4.303$.

The evidence regarding the value to be placed on observations with excised roots will be briefly discussed. Hoagland and Broyer showed by direct comparison of uptake of roots of intact barley plants and corresponding excised roots that the amount of salt absorbed by roots excised from low-salt plants was almost as large as the amount absorbed by entire plants (during a period of 7 hours), while entire high-salt plants had a much higher absorbing capacity than the corresponding excised root systems. This was presumably because the translocation of salt from root to shoot permitted more salt to enter the root system. Despite the influence of the shoot, however, they found that more salt was absorbed by the excised root system of the low-salt plants than by the high-salt plants, even though the latter were much larger. If these observations are of general application, and it seems reasonable to suppose that they are, it would mean that roots of low-salt plants (deficient in the sense used in this paper, i.e. containing a sub-normal amount of one or more mineral elements) behave rather similarly whether they are excised or intact on the plant. In other words, uptake by excised roots may be taken as a reliable guide to the existence of a deficiency in the intact plant. The fact that excised or intact roots of plants in which no deficiency exists may behave rather differently in respect of uptake does not materially affect the conclusions reached in the present paper. As regards the duration of the experiments, it may be noted that Hoagland and Broyer found that absorption of KBr by excised roots ceased after 24 hours, after which a loss of potassium occurred. However, the limitations of using excised roots must be borne in mind. Steward, Prevot, and Harrison (1942) point out that in experiments with excised roots time is an important variable, since the stimulation of the shoot is lacking and the roots eventually pass into a decline after periods which vary with their previous nutritional treatment.

Woodford and Gregory (1948) were concerned mainly with absorption by intact barley plants, but describe one experiment in which they measured the nitrate uptake and respiration rate of excised and intact roots of comparable plants. They found that the nitrate uptake of the excised roots fell after 22½ hours by 87 per cent. of the original rate. The original nitrogen and nitrate content of the tissues was not stated, but judging from the nutrient treatment received before the commencement of the experimental period, these plants were not markedly deficient in nitrogen—if they had been it is likely that there would have been a different trend subsequent to shoot removal. The initial mineral status is of great importance, and must be taken into account in considering the relative behaviour of excised and intact roots.

Steward and collaborators have put forward strong evidence for the view that rapid accumulation occurs only in active metabolizing cells and point out that the root system is not a homogeneous unit, the tissues displaying a graded activity in salt accumulation determined by the stage of development they have attained. In a later paper, however, Steward points out that low-salt roots (i.e. mineral deficient) provide an exception to this state of affairs. Here the low-salt content is due to low external concentration to which the

roots have been subjected during growth, and not to the capacity of the cells to absorb. When such root cells are supplied with abundant nutrients, they absorb up to the limit of their capacity, and growth in the sense of cell division is not a primary factor. He says: 'It is clear that this salt absorption has been deferred, and by this means segregated in time from the growth and differentiation it normally would have accompanied.' In such circumstances the gradients due to growth may be masked by the much enhanced absorption of the root as a whole (Steward, 1935).

In the experiments described in the present paper there was often loss of ions from the excised roots, if these roots had previously been grown in solutions adequately supplied with these particular ions. For instance, in Expt. II (Table II) in the roots of *Phaseolus* plants grown in a complete solution and in one lacking phosphorus, a 16 per cent. loss of potassium occurred, and also in the P-deficient solution a 13 per cent. loss of nitrogen occurred, in spite of a large increase in the phosphorus content of the root cells in this latter case. In a barley experiment (Table III) there was significant loss of N and K from roots grown in complete culture. Other experiments show a similar state of affairs. In all the experiments here reported the loss of ions took place into a solution *stronger* than that in which the plants were grown. Such large losses from tissues which are at the same time gaining in other ions does not seem to have been reported hitherto. Hoagland and Broyer (1936) found in their preliminary experiments on the factors determining rapid ion uptake by excised roots that roots with a low absorbing capacity (plants grown in complete culture solution) showed no uptake of potassium and sometimes a loss. Unfortunately they do not quote any data in support of this statement, and it is not possible to say whether the magnitude of the loss is comparable with that found in the present experiments. At any rate their general experience agrees with that of the present series of experiments, and they stress that the maximum potential for salt absorption depends not only on the presence of actively metabolizing cells, but also on the previous nutritional history and also on seasonal effects, of which carbohydrate status is likely to be one of the most important.

Since the excised root system consists of cells in all stages of development, it is impossible to say from the present experiments whether the loss of one ion and the gain of another which occurs simultaneously takes place in the same cell or cells in the same stage of development, or from different cells in different stages of development. It is possible that in the active metabolizing cells of the root which have been shown to have the greatest rate of ion absorption little loss occurs, and that the loss recorded is from cells of the cortex far removed from the chief absorbing zone. It is doubtful whether such cells can exchange ions directly with the external medium in regions where the cortex is bounded by a suberized endodermis and exodermis. Transport of ions from cell to cell in this region is not, however, excluded. Some loss of ions may occur from the cut surfaces exposed when the root system are excised. The mechanism by which loss of ions may occur is not known, but Lundegårdh (1947)

points out that 'leakage' and 'exudation' may mean outward diffusion of ions by ion-exchange processes and not simple diffusion. These possibilities must be borne in mind in formulating a theory to explain entry of ions into cells in experiments where simultaneous loss and gain of ions occurs.

Prevot and Steward's experiments (1936) using barley plants grown in complete nutrient and transferred to distilled water gave rapid uptake of both potassium ion and bromide ion in approximately equal amount. Steward terms this condition *primary salt absorption* and concluded that it is a property of actively growing metabolizing systems, whereas relatively mature cells, by virtue of a change in their external environment, readjust their salt content without gain or loss of concentration of dissolved salts—a condition termed *induced absorption*. It seems that under the condition of Steward's experiments, where all ions were restricted, a general mineral deficiency existed and when a solution of KBr was supplied it would be expected that these ions would enter the cells in more or less equal proportions. Steward, however, did not consider the case where a cation had been freely supplied, e.g. K^+ , and where an anion, e.g. NO_3^- , had been withheld. In these circumstances, as the present results show, there is uptake of NO_3^- but no change or often loss of K^+ . It seems, therefore, that the conception of primary absorption needs to be modified in such cases.

In comparing the results obtained in the present work with those of Hoagland and Broyer, Prevot and Steward, and others, it is of interest to compare the technique by which the plants were grown. Hoagland and Broyer obtained low-salt plants by allowing the culture solution to become exhausted by the growth of the plants. Prevot and Steward obtained low-salt roots by varying the period in which the roots of the plants were exposed to distilled water alone, while in the present investigation the plants were grown in solutions deficient in a particular element and strictly speaking cannot be referred to as low-salt roots, but rather as deficient roots. The authors mentioned also refer to their roots grown in this manner as 'high carbohydrate' roots. They give no figures in support of this, which is rather surprising considering the emphasis placed on this statement. It is, however, probably correct, as it is almost certain that plants grown under the conditions they employed would be nitrogen deficient and have a high carbohydrate content. A moderate deficiency of the other common elements would probably not affect the carbohydrate content either way to any large extent.

The fresh weights of the roots were determined in the present experiments before and after treatment, by centrifuging for a standard time of 2 minutes, and in all cases where the plants were grown in water culture, except in the —P treatments of Expt. II (*Pha. eolus*), there was a loss of fresh weight after treatment up to 8.9 per cent. of the initial fresh weight. Hoagland and Broyer (1936) report that in their experiments the fresh weights altered very little during the absorption period, although slight increases occurred in certain experiments in which a large amount of salt was absorbed. Unfortunately they give no figures in support of these statements.

In the experiment (No. IV) in which barley roots were treated with culture solution containing sucrose, the phosphorus and nitrogen uptake was greater than in the absence of sucrose. Roots in the presence of sucrose maintained their potassium content, while in its absence there was considerable loss. Prevot and Steward (1936) describe an experiment in which an external solution of 2 per cent. glucose was applied to absorbing roots; and they found that the low total absorption of 'high-salt, low-sugar roots' was raised to almost exactly that of low-salt roots. These authors were also able to raise the total absorption of secondary roots of *Vicia faba* by application of external sugar. Such results are in accord with those of Expt. IV and emphasize the importance of the carbohydrate status of the roots in absorption studies. The experiments of Broyer and Hoagland (1943) are also relevant in this respect. They found with winter-grown barley plants the uptake of ions was no greater during a 24-hour period than during a 12-hour daylight period—the absence of absorption during the dark period being ascribed to depletion of carbohydrate. Eaton and Joham (1944) showed that removal of flower-buds and bolls from cotton plants caused movement of carbohydrate to the fibrous roots and the amount of bromide absorbed by these greatly increased compared with normal plants. Went (1944), using radio-active phosphorus, showed that in tomato plants twice as much phosphorus reached the leaves during the daytime as during the night, and that phosphorus uptake during the day was halved by illumination during the previous night—again suggesting a relationship between uptake and carbohydrate supply.

The observation in Expt. IV that the presence of 2 per cent. sucrose maintained the potassium content of the excised roots while in the absence of sucrose there was a loss, suggests that low carbohydrate may be a factor in those experiments in which loss of ions occurred. It is apparent, however, that this cannot be the complete explanation since uptake of phosphorus occurred in the absence of external sucrose. It would appear that both carbohydrate status and the initial concentration of a particular ion in the root are at least two of the factors which have to be taken into account.

The results obtained suggest that the low-salt condition, postulated by Hoagland and Broyer (1936), is not necessarily the primary requisite for absorption of a particular ion. In most of the experiments here described the roots were almost certainly high in total salts. The condition determining absorption of a particular ion appears rather to be that a deficiency of that ion should exist in the cells.

SUMMARY

1. An apparatus is described by means of which the absorption of ions from a complete nutrient solution of constant composition by excised root systems of plants, grown under known nutrient deficiencies, may be measured in standard conditions of aeration and temperature. Results of some preliminary experiments are described.

2. It was found that the roots readily absorbed the element in which they

were deficient, but tended to lose those elements which were already present in normal amounts.

3. There was almost invariably a loss in fresh weight of the roots after the absorption period and also a loss in dry weight. This loss appears to be complex and is partly attributable to loss of respiratory material.

4. The addition of 2 per cent. sucrose to the solution from which the root systems of phosphorus-deficient barley plants were absorbing increased the nitrogen and phosphorus contents of the roots and maintained the potassium content, while in absence of sucrose only the phosphorus content increased, but this increase was significantly less than in the presence of sucrose.

5. It was shown that roots excised from plants growing in soil were capable of absorbing phosphorus or nitrogen—elements in which they were apparently deficient.

6. The interpretation of data obtained from excised roots is discussed, and it is concluded that excised roots from plants grown in complete nutrient are not likely to behave in the same way, as regards absorption, as corresponding roots of intact plants, but that roots grown under conditions of deficiency will behave rather similarly whether excised or intact. This fact provides a potential method for diagnosing and evaluating nutrient deficiencies.

7. The low-salt condition of roots postulated by Hoagland and Broyer is not necessarily the primary requisite for rapid absorption of a particular ion. It is rather that the roots should be deficient in that ion. The roots could be high in other salts.

LITERATURE CITED

- BROYER, T. C., and HOAGLAND, D. R. (1943). Metabolic activities of roots and their bearing on the relation of upward movement of salts and water in plants. *Amer. J. Bot.* **30**, 261-73.
- DONEEN, L. D. (1932). A micro-method for nitrogen in plant material. *Plant Physiol.* **7**, 717-20.
- EATON, F. M., and JOHAM, H. E. (1944). Sugar movement to roots, mineral uptake, and the growth cycle of the cotton plant. *Ibid.* **19**, 507-18.
- GREGORY, F. G., and WOODFORD, E. K. (1939). An apparatus for the study of the oxygen, salt and water uptake of various zones of the root, with some preliminary results with *Vicia faba*. *Ann. Bot.*, N.S., **3**, 147-54.
- HOAGLAND, D. R., and BROYER, T. C. (1936). General nature of the process of salt accumulation by roots with description of experimental methods. *Plant Physiol.* **11**, 471-507.
- ISMAIL, A. M., and HARWOOD, H. F. (1937). Determination of potassium as potassium-silver cobaltinitrite. *Analyst*, **62**, 443-52.
- LUNDEGÅRDH, H. (1947). Mineral nutrition of plants. *Ann. Rev. Biochem.* **16**, 503-28.
- MACHLIS, L. (1944). Influence of some respiratory inhibitors and intermediates on respiration and salt accumulation of excised barley roots. *Amer. J. Bot.* **31**, 183-92.
- MARKHAM, R. (1942). A steam distillation apparatus suitable for micro-kjeldahl analysis. *Biochem. J.* **36**, 790-1.
- MATTSON, S., ERIKSSON, E., VAHTRAS, K., and WILLIAMS, E. G. (1949). Phosphate relationships of soil and plant. I. Membrane equilibria and phosphate uptake. *Ann. Agr. Coll. Sweden*, **16**, 457-84.
- PIPER, C. S. (1942). *Soil and Plant Analysis*. Adelaide.
- PREVOT, P., and STEWARD, F. C. (1936). Salient features of the root system relative to the problem of salt absorption. *Plant Physiol.* **11**, 509-84.
- STEWART, F. C. (1935). Mineral nutrition of plants. *Ann. Rev. Biochem.* **4**, 519-44.
- , PREVOT, P., and HARRISON, J. A. (1942). Absorption and accumulation of rubidium bromide by barley plants. Localization on the roots of cation accumulation and of transfer to the shoot. *Plant Physiol.* **17**, 411-21.

- VIETS, F. G. (1942). Effects of Ca and other divalent ions on the accumulation of monovalent ions by barley root cells. *Science*, N.S., **95**, 486-7.
- (1944). Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. *Plant Physiol.* **19**, 460-80.
- WENT, F. W. (1944). Plant growth under controlled conditions. III. Correlation between various physiological processes and growth in the tomato plant. *Amer. J. Bot.* **31**, 597-618.
- WILLIAMS, R. F. (1948). The effect of phosphorus supply on the rates of intake of phosphorus and nitrogen and upon certain aspects of phosphorus metabolism in gramineous plants. *Austral. J. Sci. Res.* **1 B**, 333-61.
- WOODFORD, E. K., and GREGORY, F. G. (1948). Preliminary results obtained with an apparatus for the study of salt uptake and root respiration of whole plants. *Ann. Bot.*, N.S., **12**, 335-70.

Oxidative Assimilation of Glucose by *Scenedesmus quadricauda*

F. J. TAYLOR

Botany Department, University College, Leicester

SUMMARY

The utilization of glucose by *Scenedesmus quadricauda* has been studied by manometric methods. With small amounts the oxidation only goes to about 16 per cent. of completion, the remainder of the glucose being built up into an acid-hydrolysable polysaccharide which is not starch, and which is built up from a hexose other than fructose.

The extent of the oxidation is not affected by sodium azide or 2:4-dinitrophenol, but can be varied by altering the pH of the suspension medium; more oxygen is absorbed per unit weight of glucose at pH 8 than at pH 5.

It is suggested that there is no fixed relation between the two processes of oxidation and synthesis, as has previously been suggested, but that the glucose, after phosphorylation, saturates the oxidation system, and the excess hexose-phosphate is built up into a polysaccharide.

Mannose is also utilized in the same way, but at a slower rate, and less oxygen is used up compared with glucose. Galactose gives only a slight stimulation of the respiratory rate, and fructose, sucrose, and lactose are not absorbed.

INTRODUCTION

THE effect of glucose and other organic carbon substrates on the growth of green algae in the light and in the dark has been investigated by several workers. Pearsall and Loose (1937), for example, have studied the growth of *Chlorella vulgaris* in glucose solution in the light in very great detail, and Pearsall and Bengry (1940) its growth in relation to light intensity. Bristol-Roach (1926, 1928) has examined *Scenedesmus costulatus* var. *chlorelloides* in some detail, especially with regard to the effects of various carbohydrates on growth. However, at the moment there appears to be very little information on the actual mechanism of utilization of such added substances, and this investigation was aimed at examining the effect of glucose on the respiration of *Scenedesmus quadricauda*.

The effect of glucose on the respiration of green algae was first investigated by Emerson (1927), who found that the addition of glucose to *Chlorella pyrenoidosa* resulted in the rate of respiration being increased fourfold, and this increase was independent of the glucose concentration over the range 0.25–4.0 per cent. This increased rate of respiration was inhibited by about 50 per cent. with 10^{-4} M cyanide, which concentration had no effect on the endogenous respiration beyond increasing it slightly.

Hydrogen sulphide and carbon monoxide affected the rate of respiration in glucose solution in a similar manner, and also had no effect on the endogenous respiration. Fructose, mannose, and galactose also accelerated the respiratory

rate by three to four times, but sucrose, arabinose, glycine, dioxycetone, mannitol, and lactic acid had little effect. Genevois (1927, 1929) extended these observations to a range of algae and substrates, but his work was only qualitative.

Myers (1947) studied the oxidative assimilation of glucose by *Chlorella pyrenoidosa*, and found that when small amounts (5μ mol) of glucose were added to the algae, the rate of respiration increased, but the oxygen uptake was not sufficient for complete oxidation of the glucose. The results, assuming that the endogenous respiration is completely suppressed in the presence of added glucose, indicated that only one-sixth of the glucose was oxidized, and Myers suggested, though without an experimental basis, that the remaining five-sixths were built up into a storage substance according to the following equation:



Further work (Myers, 1949) showed that the oxygen/glucose ratio was only unity for starved cultures. With fresh cultures, ratios of the order of 1.4–1.5 were found, and Myers suggested that in the case of these cells the endogenous respiration is not completely suppressed. The R.Q. rises during glucose oxidation as the reaction proceeds (Myers and Cramer, 1948), eventually reaching values of up to 1.65. It appears, therefore, that the original equation produced by Myers is not borne out by his further work, especially as direct measurements of the cellular carbon showed that only 50 per cent. of the glucose is assimilated by fresh cells.

There are a number of differences between Myers's results and those reported below, and it appears that in part they are due to differences in experimental procedure. Myers suspended his cells in Knop's solution, whereas the results described below were carried out in phosphate buffer.

EXPERIMENTAL METHODS

Scenedesmus quadricauda (Turp.) Breb., used in these experiments, was of the same strain as that used in other investigations at Uppsala (e.g. Österlind, 1949; Rodhe, 1948). It was grown in bacteria-free culture in medium C of Österlind (1949), in which it is completely autotrophic.

Two series of experiments were carried out, the first comprising most of the work was done in the Institute for Physiological Botany, Uppsala, Sweden, and the second, involving most of the analytical work, in the Department of Botany, Sheffield University. The technique followed was the same in both cases, but the conditions of illumination differed somewhat. The Uppsala cultures were incubated in the photothermostats described by Åberg (1943, pp. 27–8) kept at 25°C ., and illuminated by a 500-watt incandescent lamp and a mercury vapour lamp, HgH 1,000, together giving a light intensity of 2,200 lux. The Sheffield cultures were illuminated by a 500-watt water-cooled incandescent lamp at summer room temperature (20° – 25°C .), giving a light intensity of 1,800 lux. The only difference noted between the two sets of cultures was that the Sheffield cultures grew at a slightly lower rate.

Each culture consisted of 1 litre of culture solution in a 2-litre conical flask. Cultures were used after 6 or 7 days in Uppsala and after 10 days in Sheffield. The yield in both cases was about 200 mg. dry weight of algae per litre.

Measurements of the rate of respiration were carried out in a Warburg micro-respirometer as described by Dixon (1943) and Umbreit, Burris, and Stauffer (1945), using Warburg's 'direct' method. In order to exclude light the manometers were covered with a specially constructed box, and measurements with a photo-electric cell in the position occupied by the flasks did not detect any light entering when the box was in position. The rate of shaking of the manometers was such that an increase in the rate did not stimulate the gas exchange, so that diffusion of oxygen was not limiting the rate of respiration. The water-bath was maintained at $25^{\circ} \pm 0.05^{\circ} \text{C}$.

When a culture was required for use the cells were centrifuged off at 2,000–2,500 r.p.m. and washed with distilled water before being suspended in the experimental medium, 3 ml. of cell suspension being used in all cases. All substances added from the side arms were first dissolved in the same solution as was used for the experimental medium. In cases other than those where a definite quantity of added substance was used, the concentrations are expressed as the final concentrations in the flask.

At the end of the experiment the contents of each flask were washed into a filter crucible, sucked dry, and dried at 102°C . overnight for the dry weight determination.

Some of the experiments were carried out on fresh cultures and others on cultures which had been kept in the dark at 25°C . for 24 or 48 hours previous to use. There appeared to be no difference between the two cultures except that the starved cultures had a lower rate of endogenous respiration.

The agreement between replicates of the same culture was within ± 5 per cent., and in the few cases where the agreement was worse than this the results have been discarded. All cultures were examined before use for visual signs of contamination. No special precautions were taken to ensure the sterility of the Warburg flasks, but very few of the curves showed any form which was suspect of contamination.

ANALYTICAL METHODS

The cells were prepared for analysis by washing out the Warburg flask into a filter crucible and sucking dry. The cells were then transferred to a small flask with a minimum of water and placed in an oven at 95°C . for half an hour to destroy the enzymes, as used by Pearsall and Loose (1937) for *Chlorella*. In the case of the acid-hydrolysed cultures, the cells were washed into a boiling tube and the acid added immediately without any preliminary heating.

The alcohol-extracted cultures were washed into small round-bottomed flasks with 70 per cent. alcohol and placed in a boiling water-bath for 15 minutes before being cooled and left overnight. The next day the alcohol was removed by distillation under reduced pressure. It was found to be essential to

remove as much alcohol as possible because ethyl alcohol interfered with the reducing sugar analyses.

The diastase-hydrolysed cells were prepared by adding 5 ml. of a solution of commercial pure diastase to the cells after heating in the oven, with a little toluene as preservative, and incubating at 25° C. for 24–48 hours.

These extracts were clarified using basic lead acetate, as recommended by Evans (1928), but using sodium phosphate as de-leading agent as suggested by Archbold and Widdowson (1931). The solution was then left overnight, and the precipitated lead and cell residue filtered off, the precipitate being washed with distilled water before being made up to a known volume.

The fats were obtained by extracting the cells with ether for 30 minutes on a boiling water-bath and leaving overnight. The ether extract was then evaporated to dryness and weighed.

Reducing sugars were estimated by Hanes's (1929) modification of Hagedorn and Jensen's method, and the results are reported in terms of glucose. At least two replicates were carried out on each sample, and they agreed to within 0.1 ml. thiosulphate. The calibration curve agreed with that published by Hanes except that the curve remained linear up to 4 mg. glucose.

Fructose was determined separately by the method of Roe (1934) and pentose by the method of Mejbaum (1939). The depth of colour in these two methods was compared in a Spekker photo-electric absorptiometer.

EXPERIMENTAL RESULTS

1. *Characteristics of the respiratory rate*

The advantages which accrue from working under conditions below pH 5.5 in the measurement of carbon dioxide output and respiratory quotients, made it obvious that the best experimental medium to use was a buffer solution of this pH. Accordingly the oxygen uptake was measured in M 15 phosphate buffers at pH 5.5 and higher. The results are presented in Table I and it can be seen that although the oxygen uptake at pH 5.5 is only about half of the uptake at pH 7, it is fairly high, and all further experiments were carried out at pH 5.5. The increase in the rate of oxygen uptake near pH 7 is presumably due to the effect of the hydroxyl ion, as noted by Warburg (1930).

TABLE I.

The effect of M 15 phosphate buffers of different pH on oxygen uptake

pH	5.56	6.47	6.95	7.44
Oxygen uptake in μ l. per 3 hours per 10 mg. dry weight	90.1	132.5	194.1	193.9

The effect of different concentrations of phosphate buffer at pH 5.56 on oxygen uptake

Concentration	M/15	M/30	M/60	Distilled water
Oxygen uptake in μ l. per 3 hours per 10 mg. dry weight	97.3	123.8	124.7	64.1

Experiments were next carried out, using different concentrations of phosphate buffer at pH 5.5, and these results are also shown in Table I. Since the rates of oxygen uptake in M/30 and M/60 phosphate were not significantly different, the former concentration was subsequently used.

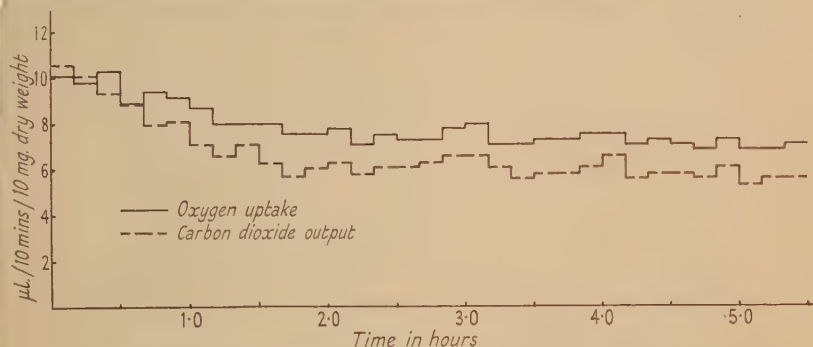


FIG. 1. Rate of respiration of fresh culture of *Scenedesmus* in phosphate buffer at pH 5.5.

As might be expected, the rate of respiration slowly declines with time, and this is shown in Fig. 1, where it will be seen that the rate falls fairly rapidly at first. This is found with fresh material, but not with cultures which have been kept in the dark, suggesting that the initial high rate is due to the oxidation of the immediate products of photosynthesis or the more easily available storage products. This is confirmed by the observation that the rate of respiration after a period of illumination in the manometer flasks is much higher than the rate before illumination. The rate then decreases at a much slower rate, and at times is nearly constant with respect to time. This can be interpreted as the oxidation of the hydrolysed food reserves, the rate of hydrolysis limiting the rate of oxidation. In cultures which have been left in the dark for 24 hours the rate is about two-thirds of that obtained with fresh cells.

The normal respiratory process results in a R.Q. of 0.85–0.90, and the Q_{O_2} value at the beginning of the experiment is usually about 7.0–7.5, falling to about 5.0–6.0 after 6 hours; the value for a culture left in the dark for 24 hours is 3.5–4.0. This is a much higher rate than that which was observed by Genevois (1929) for the respiration of certain green algae in mineral solution. For these the Q_{O_2} varied from 1.0 to 1.7, higher values, comparable with those obtained with *Scenedesmus*, only being reached on the addition of glucose. This is due to the fact that Genevois grew his algae under relatively low light intensities in order to reduce the effect of storage substances in the cell, which accounts for the low rate.

The value obtained with *Scenedesmus* is much more nearly comparable with the values obtained by Myers (1947) for *Chlorella*. Using the conversion data of cell volume in terms of dry weight (Myers 1946), this comes to a Q_{O_2} of about 5.0 in Knop's solution at pH 6.8.

The effect of acetate buffer of comparable pH on the rate of respiration was

tried. Boswell and Whiting (1938) showed that acetate buffer had a depressor effect on the rate of respiration of slices of potato and other tissues at pH 5.5, and a similar depressor effect was noticed in *Scenedesmus* (Table II). However, it is interesting to note that Algeus (1946) has found that several of the Chlorococcales, including *Scenedesmus quadricauda*, grow much better in an inorganic culture medium containing sodium acetate at pH 6.5 than in the normal culture solution, whether kept in the light or in the dark.

TABLE II

The effect of acetate buffer on gas exchange, $\mu\text{l.}/3 \text{ hrs.}/10 \text{ mg. dry weight, at low pH}$

	Phosphate buffer pH 5.56 M/30	Acetate buffer pH 5.25	
		M/15	M/30
Oxygen uptake	136.7	41.8	38.8
Carbon dioxide output	126.1	43.8	20.4

2. Oxidation of glucose

The addition of M/100 glucose to *Scenedesmus* cells results in an immediate increase in the rates of oxygen uptake and carbon dioxide output, a constant level being reached which eventually, however, slowly declines with time. The rate of respiration with added glucose is rather more than twice that without glucose, but a fairly constant level is reached irrespective of the original rate. Thus the rate is usually about 16–17 $\mu\text{l. oxygen}/10 \text{ mg. dry weight}/10 \text{ minutes}$, and this is achieved whether the addition is made to fresh cells with a corresponding rate of 7 $\mu\text{l.}$ or to starved cells with a rate of 4–5 $\mu\text{l.}$ The rate of oxygen supply, as determined by the shaking of the flasks, did not cause this limitation of oxygen uptake, since it was found possible to augment the glucose respiration by the addition of certain substances (e.g. methylene blue).

Since Myers (1947) found that *Chlorella* is capable of oxidatively assimilating glucose, definite amounts of glucose were added to cell suspensions, and the increase in gas exchange noted. On the addition of glucose the rates of oxygen uptake and carbon dioxide output both rose rapidly, and after remaining constant, fell off, rapidly at first, and then more gradually. However, the oxygen used up and the carbon dioxide liberated, only amounted to about 16 per cent. of that required for complete oxidation of the glucose. A typical experiment is shown in Fig. 2, where four different amounts of glucose were oxidized by samples from the same culture. Since the duration of the high rates is roughly proportional to the amounts of glucose added, it seems reasonable to assume that the fall is due to the complete utilization of the glucose.

Actual proof that the break in the curve coincides with the utilization of all the glucose was obtained by filtering off the cells and analysing the suspension medium for glucose as soon as the break occurred. None, or only very small amounts, remained, indicating that almost complete absorption had occurred. That there was no appreciable accumulation of free glucose in the cell was

shown by comparing the reducing sugar content of alcohol extracts of the cells supplied with glucose with control cells (see Table VII, p. 315). Thus all the glucose must be either oxidized completely or must undergo some other transformation.

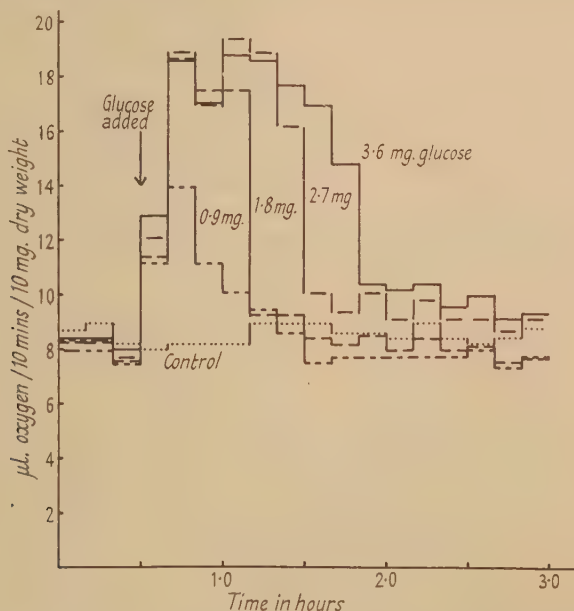


FIG. 2. Effect of different amounts of glucose on the oxygen uptake of *Scenedesmus*.

In making an estimate of the amount of oxygen used up in the oxidation of the glucose, the problem arises as to how much allowance, if any, must be made for the normal endogenous respiration. There are three possibilities. Firstly, the glucose is oxidized in addition to the normal respiratory substrate; secondly, following the addition of glucose, only the glucose is oxidized, the endogenous respiration being reduced to zero; and thirdly, part only of the endogenous respiration is cut down. This problem, moreover, is incapable of being settled by direct experiment by manometric techniques. Barker (1936) used an empirical method to suggest that the endogenous respiration is almost completely suppressed in the presence of added substrate, and all later workers have assumed this to be the case.

Barker suggested that the most accurate correction for the endogenous respiration was the one which, when applied to several experiments, or to several flasks in the same experiment, gave the most constant value for oxygen absorbed per unit quantity of substrate oxidized. Table III shows the results of a series of experiments where the results have been calculated on the assumption that the endogenous respiration is completely suppressed in the

presence of added glucose, while in Table IV the same data are presented, calculated on the assumption that the endogenous respiration is unaffected. It will be seen that the first set of data give much more consistent values for the volume of oxygen per unit weight of glucose. Further, in the second set of data, the R.Q. varies considerably, from 0.67 to 1.48, whereas in the first set it is consistent, just below unity.

TABLE III

Ratios of oxygen absorbed and carbon dioxide liberated to glucose oxidized, assuming no endogenous respiration

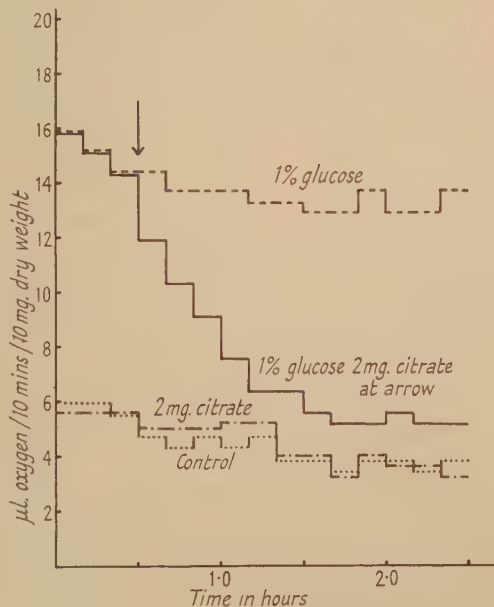
Expt. No.	Glucose added, mg.	μ l. oxygen absorbed	μ l. carbon dioxide liberated	Ratio oxygen/0.9 mg. glucose	Mols oxygen/mol. glucose	R.Q.
19	0.9	120.8	117.0	120.8	1.08	0.98
20	0.9	124.1	110.6	124.1	1.11	0.89
22	0.9	126.3	124.1	126.3	1.13	1.02
26	0.9	110.8	101.4	110.8	0.99	0.92
27	0.9	119.3	—	119.3	1.07	—
22	1.8	242.5	242.5	121.2	1.08	1.00
26	1.8	206.9	195.1	103.4	0.92	0.95
27	1.8	214.3	—	107.1	0.96	—
26	2.7	329.3	313.1	109.8	0.98	0.95
27	2.7	315.6	—	105.2	0.94	—
20	3.6	425.4	393.7	106.4	0.95	0.93
27	3.6	433.8	—	108.5	0.97	—
Mean				113.6	1.01	0.94
Standard deviation				10.2		
Coefficient of variation				8.98		

TABLE IV

Ratios of oxygen absorbed and carbon dioxide liberated to glucose oxidized, assuming complete endogenous respiration

Expt. No.	Glucose added, mg.	μ l. oxygen absorbed	μ l. carbon dioxide liberated	Ratio oxygen/0.9 mg. glucose	Mols oxygen/mol. glucose	R.Q.
19	0.9	95.3	115.0	95.3	0.85	1.21
20	0.9	80.8	120.0	80.8	0.72	1.48
22	0.9	76.0	50.6	76.0	0.68	0.67
26	0.9	55.4	48.6	55.4	0.49	0.88
27	0.9	37.8	—	37.8	0.34	—
22	1.8	134.1	140.2	67.0	0.60	1.05
26	1.8	127.8	181.5	63.9	0.57	1.42
27	1.8	105.3	—	52.7	0.47	—
26	2.7	223.0	227.1	74.3	0.66	1.02
27	2.7	145.2	—	48.4	0.43	—
20	3.6	306.1	307.5	76.5	0.68	1.01
27	3.6	204.5	—	51.1	0.46	—
Mean				64.9	0.58	1.09
Standard deviation				16.4		
Coefficient of variation				25.3		

One drawback to the use of Barker's method is that it cannot distinguish concentration effects. However, with *Scenedesmus*, inspection of the data shows no pronounced concentration effects. Since the coefficient of variation, assuming that the endogenous respiration is completely suppressed, is about one-third of that assuming the endogenous respiration to be unaffected, it seems reasonable to conclude that the glucose does suppress the oxidation of the normal respiratory substrate.



glucose, on phosphorylation, is incorporated into the normal respiratory system in place of the hexose phosphate which results from hydrolysis of the reserve substances. Since the same system is used for the oxidation of both the added glucose and the reserve carbohydrates, there can be no real distinction between endogenous and exogenous respiration. However, it may be desirable to speak of the oxidation of the normal cell carbohydrates as endogenous respiration.

It also appears that a good supply of oxygen is necessary for the uptake of glucose by the cells. In the Warburg apparatus, where there was no limitation of oxygen supply, 3.6 mg. glucose were absorbed in a matter of 2 hours. In another shaker working at a slower rate in a water-bath at the same temperature, absorption of this amount of glucose took 5 to 6 hours whilst in unshaken flasks very little glucose was absorbed after 6 hours.

3. *Effect of glucose concentration*

No strictly comprehensive series of experiments were carried out to determine the effect of different concentrations of glucose on the amount of oxygen absorbed, but the data do not suggest much variation, though more oxygen is apparently used up with smaller amounts of glucose. This may be due to the fact that with small amounts of glucose (0.9 mg.) there is not enough glucose present to saturate the enzyme surfaces, and consequently there is a certain amount of endogenous respiration going on as well, especially at the very beginning and towards the end of glucose oxidation. This is borne out by the form of the curve during the oxidation of 0.9 mg. glucose (Fig. 2); the curve rises steeply and falls immediately, so that the oxidation is over in a relatively short time, and the highest oxidation rate reached is lower than with larger amounts of glucose. In some of these experiments the exact location of the break is made with some uncertainty, as it is not so clear as with larger amounts of glucose.

In experiments where some of the algae had been respiring in 1 per cent. glucose for a period of several hours, the rate of respiration of these algae showed a decline after about 2 hours, although still remaining well above the control rate without glucose (see Fig. 3). This is possibly due to the accumulation of the products of assimilatory activity.

4. *Effect of the pH of the suspension fluid*

The effect of pH on the rate of endogenous respiration has already been considered (p. 304). The effect of phosphate buffers of different pH on the amount of oxygen used up in the oxidation of 3.6 mg. glucose was also investigated. The results of one of these experiments are shown in Fig. 4, where it will be seen that an increase in the pH results in more oxygen being consumed per unit of glucose added. There appears to be a linear relation between the rate of respiration just prior to tipping the glucose and the amount of oxygen utilized in oxidizing the glucose (Fig. 5; correlation coefficient +0.985). This shows that an alteration in the pH of the external medium

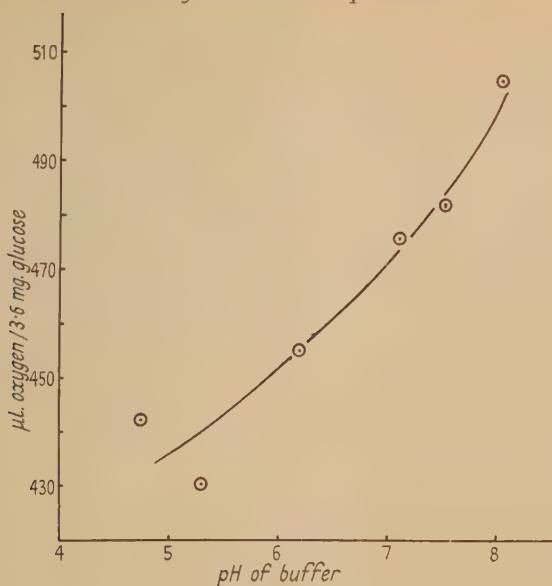


FIG. 4. Amount of oxygen used in the oxidation of 3.6 mg. glucose at different pH values.

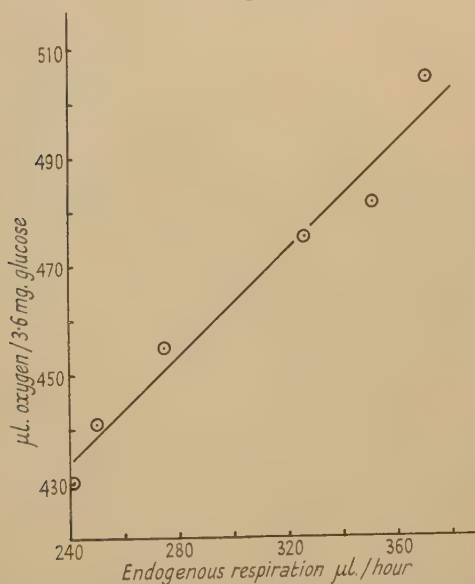


FIG. 5. Amount of oxygen used in the oxidation of 3.6 mg. glucose compared with the endogenous respiration at different pH values. (Data of Fig. 4.)

has a similar effect on both the endogenous and the glucose respiration, suggesting that the two processes are identical.

The time taken to reach the break in the oxygen uptake curve with equal amounts of glucose is the same at all the pH values. This means that the glucose is being absorbed at the same rate in all cases, but since more oxygen is being used up during this period at higher pH values, it follows that more glucose is being oxidized at a high pH than at a low pH. Thus there is less glucose available for assimilation at a high pH. This fact, that the ratio of oxygen consumed to glucose utilized can be varied by means of a variation in the acidity of the medium, suggests that the two processes of oxidation and assimilation are not so closely linked as previous investigators have suggested.

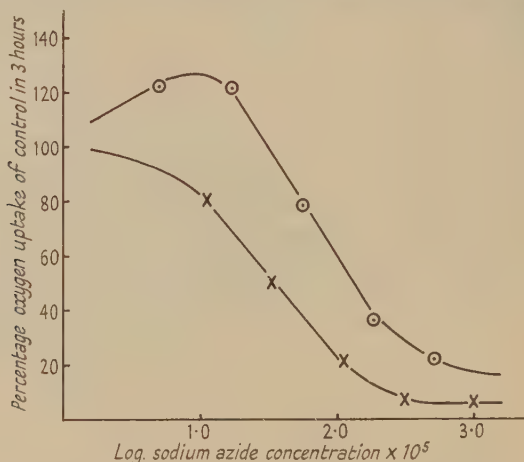


FIG. 6. Effect of sodium azide concentration on the oxygen uptake of *Scenedesmus* with ($\times$) and without ($\circ$) added glucose.

5. Effect of respiratory inhibitors

Clifton (1937) demonstrated that the addition of certain inhibitors to various bacteria at concentrations which had no effect on the rate of respiration resulted in complete oxidation of added substrates. Therefore the effects of certain of these inhibitors were tried on *Scenedesmus*.

Sodium azide in low concentrations does not inhibit, but rather stimulates, the rate of endogenous respiration up to concentrations of about 7.5×10^{-3} M (the actual concentration depending upon the number of cells present in the flask). Above this concentration, inhibition sets in, and this follows a logarithmic curve until high concentrations are reached, when the curve flattens out and a small amount of azide-resistant respiration is left (Fig. 6). This azide-resistant respiration has a Q_{O_2} of 1.7 and a high R.Q. (1.5–1.6) compared with the normal endogenous R.Q. In common with other organisms this azide inhibition is reversible, the original rate of respiration being regained on washing with water.

With 1 per cent. glucose the respiration is also inhibited by azide, but there is no preliminary stimulation with low concentrations. Inhibition again follows a logarithmic curve until an azide-resistant respiration is reached, which has the same absolute value as that obtained by the action of azide on the endogenous respiration. This confirms Commoner's (1940) conclusion with cyanide, that the cyanide-resistant respiration rate is independent of any added substrate.

When 3.6 mg. glucose was added to *Scenedesmus* cells respiring in 7.5×10^{-3} M azide, which concentration had no effect on the endogenous respiration, the rate of glucose oxidation was reduced, but the total oxygen absorbed was still 16 per cent. of that required for complete oxidation of the glucose.

Similar experiments were carried out with 2:4-dinitrophenol. With concentrations of up to 10^{-3} M, dinitrophenol produced only a stimulation of the respiratory rate, whether glucose was present or not. Concentrations of 10^{-4} M dinitrophenol had no effect on the amount of oxygen absorbed in the oxidation of 3.6 mg. glucose over the control.

6. Nature of the assimilation product

Since the analyses of the contents of the manometric flasks showed that all the glucose had been absorbed by the cells, a number of experiments were carried out to determine the products of metabolism other than carbon dioxide. The results of the experiment shown in Table V indicate that all the products are retained by the cells. In this experiment 3.6 mg. glucose were added to the respiring cells, and as soon as the increased oxygen uptake ceased the cells were filtered on to a filter crucible, and the suspension medium was collected in a small flask, together with a little distilled water, which was used to wash out the flasks. The crucibles and flasks were then dried overnight at 102° C. It will be seen that the weight of suspension medium from the glucose flasks does not differ significantly from the control flasks, but that the dry weights of the cells have increased by an amount (3 mg.) which would be expected if only one-sixth of the glucose had been oxidized.

In order to confirm that the glucose had been changed chemically in the cell, a comparison was made of the reducing sugar contents of control cells with cells which had oxidatively assimilated 3.6 mg. glucose. The results of this are shown in the lower part of Table V, and it will be seen that there is no significant increase in the cells supplied with glucose, showing that all the glucose is absorbed into the cell and either oxidized or converted into some non-reducing substance.

Neither was there any significant increase in the dry weight of the ether-soluble extracts from the two types of cell (Table VI), indicating that the glucose was not converted into a fatty substance or lipid.

After hydrolysis of the cells on a water-bath for three hours in N. HCl, however, a significant increase in the reducing content of the glucose cells over the control cells was detected. A number of these experiments are shown in Table VII, where it will be seen that, although there is a definite increase, it is not so great as would be expected from the manometric experiments.

TABLE V

Increase in dry weights during oxidation of 3.6 mg. glucose

	Weight of dried alga (mg.)	Weight of suspension residue (mg.)	Total increase in dry weight (mg.)
Flask 7 (Control).	31.3	10.4	—
Flask 3 . . .	34.3	10.6	3.2
Flask 1 . . .	34.1	10.4	2.8
Flask 8 (Control).	31.3	14.5	—
Flask 4 . . .	34.6	14.3	3.1
Flask 2 . . .	34.3	14.4	2.9

Reducing sugar contents of Scenedesmus cells after oxidative assimilation of 3.6 mg. glucose

Expt. No.	Reducing sugar, mg. glucose equivalents		
	Glucose in medium	Cells without added glucose	Cells with added glucose
120 . . .	0.15	0.86	0.80
121 . . .	0.00	0.50	0.54

TABLE VI

Weights in mg. of ether extracts of Scenedesmus cells after oxidative assimilation of 3.6 mg. glucose

	Cells without added glucose		Cells with added glucose
Flask 20 .	2.0	Flask 19 .	2.7
Flask 17 .	2.6	Flask 21 .	2.5
	—	Flask 15 .	3.1
Mean .	2.3	Flask 18 .	2.3
		Mean .	2.6

Analyses were carried out to determine whether fructose or pentose were formed on acid hydrolysis. If the oxidation of the glucose molecule is simply a decarboxylation of one carbon atom accompanied by oxidation, the result would be a pentose. The fructose and pentose analyses showed no increase in the glucose cells over the control.

In order to test whether the storage substance is actually starch, cells, after heating in the oven for 30 minutes, were hydrolysed with a diastase solution for 2 days and the reducing sugar contents measured (Table VII). These results showed no significant increases in the glucose cells suggesting that the storage product is not starch. It remains possible that the diastase had only partially hydrolysed the product of assimilation to a dextrin, but this does not seem very likely as there is a certain amount of starch in the cells already. Moreover, a comparison of the values given in Table VII suggests that there is present in the cells a polysaccharide other than starch, and that this is identical with that which results from the assimilation of glucose. This is borne out by further analyses of starch and total acid-hydrolysable polysaccharide carried out on the same sample of cells.

TABLE VII

Increases in reducing sugar contents as mg. glucose of cells on acid-hydrolysis after oxidative assimilation of 3.6 mg. glucose and total gas exchange of samples supplied with sugar

Cells without glucose		Cells with glucose		μ l. Oxygen absorbed	μ l. Carbon dioxide liberated
Flask 17	5.4	Flask 19	8.0	423	—
Flask 18	5.2	Flask 15	7.8	—	408
Mean	5.3	Flask 20	8.1	410	—
		Flask 21	7.8	—	395
		Mean	7.9		
Mean increase 2.6 mg. Expected increase 3.0 mg.					
Flask 20	8.4	Flask 19	11.2	385	—
Flask 17	8.8	Flask 21	10.8	—	358
Mean	8.6	Flask 15	10.8	416	—
		Flask 18	11.2	—	380
		Mean	11.0		
Mean increase 2.4 mg. Expected increase 3.1 mg.					
Flask 16	7.4	Flask 14	9.6	414	—
Flask 15	7.0	Flask 11	9.6	—	377
Mean	7.2	Flask 10	9.6	416	—
		Flask 9	9.7	—	381
			9.6		
Mean increase 2.4 mg. Expected increase 3.1 mg.					

The expected increase is obtained by subtracting glucose respired from the total originally present based on the gas exchanges.

Increases in reducing sugar contents of cells on hydrolysis with diastase after oxidative assimilation of 3.6 mg. glucose

Cells without glucose		Cells with glucose		μ l. Oxygen absorbed	μ l. Carbon dioxide liberated
Flask 20	2.3	Flask 19	2.4	404	—
Flask 17	2.4	Flask 21	2.4	—	388
Mean	2.4	Flask 15	2.6	401	—
		Flask 18	2.2	—	393
		Mean	2.4		
Flask 20	4.1	Flask 19	3.8	405	—
Flask 17	3.4	Flask 21	3.8	—	378
Mean	3.7	Flask 15	4.4	400	—
		Flask 18	4.0	—	382
		Mean	4.0		

This discrepancy between the amount of glucose actually used in synthesis compared with the amount expected from manometric experiments has been observed previously in other organisms. It is presumably due to participation of glucose in syntheses of other substances not necessarily carbohydrate.

7. Effects of other sugars

The effect of the addition of some other sugars was tried. With mannose there was an increase in the rates of oxygen uptake and carbon dioxide output, but this increased rate of respiration did not reach the level obtained with glucose (Fig. 7). On the addition of mannose the rate of oxygen uptake increased fairly rapidly to a level of about $9\mu\text{l.}/10\text{ min.}/10\text{ mg. dry weight}$, and

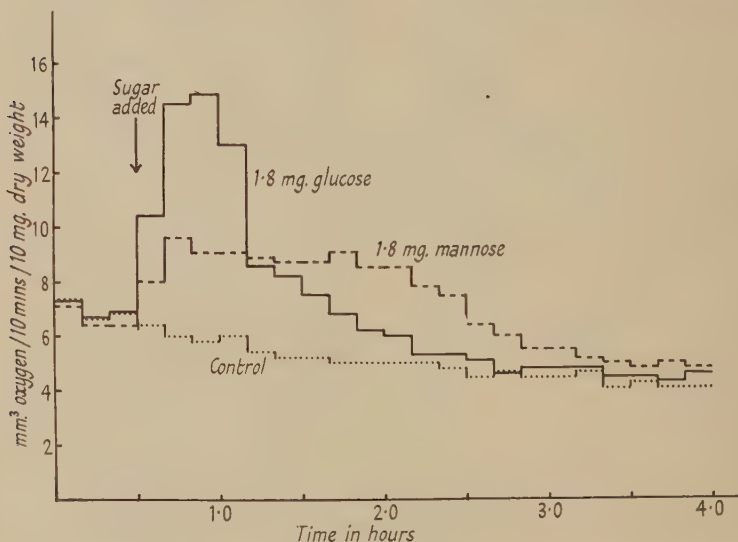


FIG. 7. Comparison of the effects of adding 1.8 mg. glucose and mannose to *Scenedesmus*.

then, after remaining steady for some time, fell off till the rate reached the level of the control. In this case it does not seem possible to suggest that the addition of the sugar causes a saturation of the enzyme surfaces, since the rate does not reach the glucose level. It therefore appears that, with mannose, oxidation of the sugar occurs in addition to the endogenous respiration—the two processes are going on simultaneously. The cause of the low rate of oxidation of mannose appears to be due to the fact that it is not absorbed very rapidly by the cells. This slow rate of absorption of mannose has previously been described for *Chlorella* by Tang (1937) and from the gut of the rabbit (Bailey and Roe 1944). It may be due to a low rate of phosphorylation (Berger, Slein, Colowick, and Cori, 1946).

Assuming that the endogenous respiration is not affected by the addition of mannose, the excess oxygen taken up and carbon dioxide evolved is shown in Table VIII. Here it is seen that the oxygen/mannose ratio is fairly constant for amounts of the order of 1.8 mg. mannose. In the one experiment where 3.6 mg. mannose were oxidized the ratio is much smaller, and here the experiment may not have run quite to completion. Further, in most cases the R.Q.

is close to unity, and it appears that here we are dealing with a mechanism analogous to the oxidative assimilation of glucose, although the oxygen/hexose ratio is lower in this case.

Two other hexoses, fructose and galactose, were tried. In the case of fructose only small values were obtained for the excess gaseous exchange (Table VIII). Different samples were tried in order to test whether this was due to impurities, but in all cases there was a slight excess which did not appear to bear any relation to the concentration of the fructose. Analyses of the suspension fluid were also carried out, when it was found that very little fructose had been absorbed by the cells after periods of 3 hours. With galactose the increases were rather greater than with fructose, but were not of the same order as the increases with glucose and mannose, being about 10–20 per cent. of the values obtained with glucose.

TABLE VIII

Excess oxygen uptake and carbon dioxide output in μ l. after the addition of mannose

Expt. No.	Mannose added (mg.)	Excess oxygen uptake	Excess carbon dioxide output	Oxygen/mannose ratio	R.Q.
79	0.9	70.5	57.9	70.5	0.82
79	0.9	62.5	52.0	62.5	0.83
86	0.9	63.0	55.7	63.0	0.88
84	0.9	63.6	62.3	63.6	0.98
78	1.8	140.3	142.1	70.1	1.00
86	1.8	127.2	125.9	63.6	0.99
100	1.8	122.9	137.6	68.8	1.10
100	3.6	214.6	199.5	53.7	0.93

Excess oxygen uptake and carbon dioxide output in μ l. after the addition of various sugars

Sugar	Amount added (mg.)	Excess oxygen uptake	Excess carbon dioxide output
Galactose	0.9	+22.2	+23.7
	1.8	+19.5	+29.6
	1.8	+33.6	+34.6
	3.6	+50.8	+65.0
Fructose	1.8	+12.9	+15.0
	1.8	+28.2	+37.3
	3.6	+6.5	+12.1
Sucrose	1.8	— 4.7	+6.1
	1.8	— 8.2	— 2.3
Lactose	1.9	— 38.0	— 58.2

The disaccharides, sucrose and lactose, were also tried. In both cases rather less oxygen was absorbed with the sugar than without. Saïd (1948) has suggested that in the case of barley seedlings and certain other plant tissues, sucrose is not absorbed directly but is first phosphorylated, probably in a similar manner to that proposed by Doudoroff, Kaplan, and Hassid (1943) for

Pseudomonas saccharophila. However, such a reaction is not likely to occur in *Scenedesmus* as there is plenty of inorganic phosphate available for phosphorylation in the suspension medium.

The addition of a hexose di-phosphate solution resulted in an increase in the rate of oxygen uptake and carbon dioxide output, but no quantitative experiments were carried out with this preparation.

DISCUSSION

The experimental data outlined above show that on the addition of glucose to *Scenedesmus quadricauda* only part of it is oxidized, the rest being built up into an acid-hydrolysable polysaccharide. It is proposed in this section to consider this process in relation to the general metabolism of the organism.

Clifton (1946) has summarized a great deal of similar work carried out with heterotrophic organisms and is of the opinion that there is a definite relationship between oxidation and assimilation, which can be expressed by a chemical equation. Myers (1947) has interpreted his results with *Chlorella* in the same way.

Although the data with *Scenedesmus* can be interpreted in a similar manner, there remains another possibility which does not appear to have been previously considered to any extent, and that is that some of the added substrate is completely oxidized and the rest is assimilated, the ratio between the two processes being dependent on the environmental conditions. In *Scenedesmus* the proportion of glucose oxidized to glucose assimilated can be altered to a certain extent by varying the acidity of the medium. With *Chlorella*, Myers's results show that the relationship between the two processes varies according to whether fresh, growing cells are used, or starved ones.

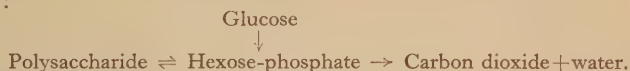
It is difficult to view the actual decomposition of the glucose in either *Chlorella* or *Scenedesmus* on the basis of Clifton's theory. In most cases so far investigated a hexose is first phosphorylated and then broken down into two triose molecules, which are then oxidized to carbon dioxide and water by some oxidation system. On Clifton's view, one third of one of these triose molecules is oxidized and the remaining two thirds, together with the other triose molecule, is assimilated. A scheme which could fit in with the observed results has been put forward by Dickens (1938) for yeast. Here oxidation and decarboxylation of a hexose-phosphate results in formation of pentose-phosphate which undergoes further oxidation and decarboxylation to give a triose-phosphate which is then oxidized as in normal glycolysis. If oxidation and decarboxylation only went as far as the pentose-phosphate, and this was built up into a pentosan, this would only require one-sixth of the oxygen for complete combustion, and would yield a R.Q. of unity. With *Scenedesmus*, however, there appeared to be no significant increase in the pentosan content of the cells with added glucose over the cells without glucose, so that this does not appear to take place.

Although no evidence has been presented to show the occurrence of a phosphorylation cycle in *Scenedesmus*, there are strong grounds for assuming

its presence from analogy with other organisms, coupled with the fact that hexose-diphosphate stimulates the rate of respiration. Emerson, Stauffer, and Umbreit (1944) have investigated phosphorylation in *Chlorella* and found that, although phosphorylation does occur, it differs in several respects from phosphorylation in other organisms, mainly in the nature of the phosphorus compounds.

It is known that during oxidation a number of energy-rich phosphate bonds are produced which are available for the phosphorylation of further hexose molecules, and it seems reasonable to suggest that they effect the phosphorylation of glucose molecules which, instead of being oxidized, are synthesized to polysaccharide with the liberation of inorganic phosphate.

Thus the following scheme connecting the various systems may be constructed:



Under normal conditions, with no added respiratory substrate, the process limiting the rate of respiration is the rate of production of hexose-phosphate from the polysaccharide. On the addition of glucose this reaction is by-passed, and the limiting reaction becomes the rate of oxidation of products of glycolysis. This is borne out by the effect of azide on oxidation. With any given concentration of azide the percentage inhibition is greater in the presence of added glucose, though the azide-resistant respiration is reached with roughly the same concentration. This effect would be obtained if, in the absence of glucose, the oxidative enzyme surfaces were not fully saturated with substrate. It seems likely that the stimulation with low concentrations of azide can be explained on a similar basis; with very dilute solutions, enough azide has not been added to cause a marked blocking of the oxidase enzyme surfaces, but these low concentrations stimulate some other enzyme system, so that the curve obtained with azide is actually a resultant of two reactions—an inhibition and a stimulation. The failure of cyanide, sulphide, and carbon monoxide to inhibit the endogenous respiration of *Chlorella* (Emerson 1927) may be explained in a similar way—not enough inhibitor had been added to cause an effect—rather than indicating the presence of two sets of oxidizing enzymes.

The finding that in *Scenedesmus* more synthesis is carried on at low pH values may be directly correlated with the phosphorylase equilibrium as demonstrated by Hanes (1940), who showed that the reaction



lies over to the right, and more starch is formed at pH 5 than at pH 7.

The hexoses glucose, mannose, and fructose are interconvertible in plant tissues by a series of trans-phosphorylases. One would therefore expect each of these hexoses to have a similar effect on assimilation and oxidation. The fact that mannose is not oxidized to such a great extent as glucose may be taken as a further indication that the hexose/oxygen ratio can be varied if it is assumed

that the two hexoses yield the same polysaccharide on assimilation. Berger, Slein, Colowick, and Cori (1946) have shown that the phosphorylation of mannose by hexokinase isolated from yeast proceeds at only 30 per cent. of the rate of glucose phosphorylation. Thus the differences observed between the effects of glucose and mannose can be interpreted as differences due to variations in the supply of hexose-phosphate to the oxidative and synthetic systems as a result of differences in the rate of phosphorylation. In any case, the mannose data cannot be represented by any simple equation on Clifton's theory.

The storage product in *Scenedesmus* does not appear to be cellulose, starch, or a disaccharide, as indicated by the data of Table VII. These data suggest that it is some polysaccharide as yet not clearly identified.

ACKNOWLEDGEMENTS

The major part of this investigation was carried out during the tenure of a post-graduate scholarship from the Swedish Institute for Cultural Relations, for which the writer is deeply indebted. The work was carried out in the Institute for Physiological Botany, in the University of Uppsala, and the writer is extremely grateful to Professor Elias Melin for placing the facilities of the Institute at his disposal, and also to Dr. Sven Österlind and other members of the Institute for much kind help. The writer is also very grateful to Mr. J. G. Boswell for kind advice and criticism, and for permission to carry out a number of manometric experiments in the Department of Botany, Sheffield University.

It is also wished to acknowledge the receipt of a grant from the Royal Society for the purchase of a Spekker photo-electric absorptiometer.

LITERATURE CITED

- ÅBERG, B. (1943). Physiologische und ökologische Studien über die pflanzliche Photomorphose. *Symb. Bot. Upsala*, **8**, No. 1.
- ALGEUS, S. (1946). Untersuchungen über die Ernährungsphysiologie der Chlorophyceen. *Bot. Notiser*, 1946, 129.
- ARCHBOLD, H. K., and WIDDOWSON, E. M. (1931). Iodometric determination of reducing sugars in the apple. *Biochem. J.* **25**, 101.
- BAILEY, E. H., and ROE, J. H. (1944). Studies of the metabolism of mannose. The influence of mannose administration on blood sugar, blood lactic acid, and liver glycogen in the adult rabbit. *J. Biol. Chem.* **152**, 135.
- BARKER, H. A. (1936). The oxidative metabolism of the colorless alga *Prototheca zopfii*. *J. Cell. & Comp. Physiol.* **8**, 231.
- BERGER, L., SLEIN, M. W., COLOWICK, S. P., and CORI, C. F. (1946). Isolation of hexokinase from bakers yeast. *J. Gen. Physiol.* **29**, 379.
- BOSWELL, J. G., and WHITING, G. C. (1938). A study of the polyphenol oxidase system in potato tubers. *Ann. Bot.*, n.s. **2**, 847.
- BRISTOL-ROACH, B. M. (1926). On the relation of certain soil algae to some soluble carbon compounds. *Ibid.* **40**, 149.
- (1928). On the influence of light and of glucose on the growth of a soil alga. *Ibid.* **42**, 317.
- CLIFTON, C. E. (1937). On the possibility of preventing assimilation in respiring cells. *Enzymologia*, **4**, 246.
- (1946). Microbial assimilations. *Advances in Enzymology*, **6**, 269.

- COMMONER, B. (1940). Cyanide inhibition as a means of elucidating the mechanisms of cellular respiration. *Biol. Rev.* **15**, 168.
- DICKENS, F. (1938). Oxidation of phosphohexonate and pentose-phosphoric acids by yeast enzymes. *Biochem. J.* **32**, 1626.
- DIXON, M. (1943). *Manometric methods*. 2nd ed. Cambridge, 1943.
- DOUDOROFF, M., KAPLAN, N., and HASSID, W. Z. (1943). Phosphorylation and synthesis of sucrose with a bacterial preparation. *J. Biol. Chem.* **148**, 67.
- EMERSON, R. (1927). The effect of certain respiratory inhibitors on the respiration of *Chlorella*. *J. Gen. Physiol.*, **10**, 469.
- STAUFFER, J. F., and UMBREIT, W. W. (1944). Relationships between phosphorylation and photosynthesis in *Chlorella*. *Amer. J. Bot.* **31**, 107.
- EVANS, D. I. (1928). Chemical studies in the physiology of apples. VII. A study of the sugars of apples with especial reference to the fructose/glucose ratio. *Ann. Bot.* **42**, 1.
- GENEVOIS, L. (1927). Über Atmung und Gärung in grünen Pflanzen. *Biochem. Z.* **186**, 461.
- (1929). Sur la fermentation et sur la respiration chez les végétaux chlorophylliens. Parts 3 and 4. *Rev. Gén. de Bot.* **41**, 49, 119.
- HANES, C. S. (1929). An application of the method of Hagedorn and Jensen to the determination of larger quantities of reducing sugars. *Biochem. J.* **23**, 99.
- (1940). The reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase. *Proc. Roy. Soc. B*, **129**, 174.
- MEJBAUM, W. (1939). Über die Bestimmung kleiner Pentosemengen insbesondere in Derivaten der Adenylsäure. *Z. physiol. Chem.* **258**, 217.
- MYERS, J. (1946). Culture conditions and the development of the photosynthetic mechanism. III. Influence of light intensity on cellular characteristics of *Chlorella*. *J. Gen. Physiol.* **29**, 419.
- (1947). Oxidative assimilation in relation to photosynthesis in *Chlorella*. *J. Gen. Physiol.* **30**, 217.
- (1949). Effect of starvation on the metabolism of *Chlorella*. *Plant Physiol.* **24**, 255.
- and CRAMER, M. (1948). Metabolic conditions in *Chlorella*. *J. Gen. Physiol.* **32**, 103.
- ÖSTERLIND, S. (1949). Growth conditions of the alga *Scenedesmus quadricauda*. *Sym. Bot. Upsala*, **10**, No. 3.
- PEARSALL, W. H., and BENGTRY, R. P. (1940). Growth of *Chlorella* in relation to light intensity. *Ann. Bot.*, N.S. **4**, 485.
- and LOOSE, L. (1937). The growth of *Chlorella vulgaris* in pure culture. *Proc. Roy. Soc., B*, **121**, 451.
- RODHE, W. (1948). Environmental requirements of freshwater plankton algae. *Sym. Bot. Upsala*, **10**, No. 1.
- ROE, J. H. (1934). A colorimetric method for the determination of fructose in blood and urine. *J. Biol. Chem.* **107**, 15.
- SAID, H. (1948). Effect of phosphorus on the hydrolysis and absorption of sucrose by plant cells. *Nature*, **162**, 496.
- TANG, P. S. (1937). Studies on the kinetics of cell respiration. VI. Respiration of *Chlorella pyrenoidosa* in the presence of lactates. *J. Cell. & Comp. Physiol.* **10**, 499.
- UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. (1945). *Manometric techniques and related methods for the study of tissue metabolism*. Minneapolis, 1945.
- WARBURG, O. (1930). *Metabolism of tumours*. Translated by F. Dickens. London, 1930.

The Rate of Water-loss from Stripped Leaves

M. A. ABOU RAYA

Botanical Department, Queen Mary College, University of London

IN the course of studying the effect of stomatal number on transpiration, the question has arisen to what extent the stomatal epidermis checks the rate of water-loss from the mesophyll cells. One means of studying this is to measure the relative loss of water-vapour from the mesophyll with the epidermal resistance removed. Two methods may be used: (a) cutting or puncturing the epidermis, and (b) stripping off the epidermis altogether. The first method was used by Darwin (1914) and later by Henderson (1926). Clearly this method could not be used here since the equivalent area of mesophyll exposed cannot be measured and no comparison can therefore be obtained with the corresponding area of intact leaf. The second method was used recently by Lewis (1948), who discusses some of the properties of the mesophyll in this connexion.

Suitable leaves for stripping experiments are found in those of succulent plants which can be stripped with great ease. *Sedum spectabile* was found to strip so easily that the whole epidermis could be removed in a few operations. In such stripping no deep-seated injury to the mesophyll layer could be observed. The epidermis comes away very readily and cleanly and free from any remnant of mesophyll cells, these latter remaining quite undamaged. A further advantage of this plant is that it has nearly equal numbers of stomata on the two surfaces of the leaves.

For measuring transpiration rate, the method first used by Glover (1941) was selected. This method has the advantage of measuring transpiration rate from single leaves under closely natural conditions. The technique is fully described in the author's paper (loc. cit.). Certain modifications were found necessary before the method was finally adapted for the present work. A full description of these modifications will be published shortly. In the present connexion only the following points need be mentioned:

- (a) The method depends upon measuring the amount of water-vapour in air which has been drawn over the leaf by the lowering in temperature of a small evaporating surface as shown by a thermocouple and mirror galvanometer.
- (b) The apparatus is constructed and calibrated so as to be capable of measuring the transpiration rates at air speeds of 5, 10, 15, and 20 litres per hour.
- (c) Special perspex leaf chambers were used which not only allowed the exposure of a maximum area of leaf surface but also permitted independent measurement of the transpiration from the two surfaces of the leaf.

EXPERIMENTAL RESULTS

Cut shoots of *Sedum spectabile* were selected and brought to the laboratory from the Chelsea Physic Garden. These shoots usually bore from six to seven healthy leaves, one of which from the middle of the shoot was used. The area of the leaf was measured by means of a planimeter and the leaf set up in the leaf chamber. The exposed transpiring areas of both surfaces were also measured and recorded. Air was then drawn over the transpiring area at the required speed and the galvanometer deflexions recorded at 5-minute intervals. This continued for half an hour with each surface, and the corresponding transpiration rates were calculated in $\text{mg. hr.}^{-1} \text{ dm.}^{-2}$. On changing the air speed, the leaf chamber with the leaf in it was disconnected from the rest of the apparatus, which was allowed to settle down at the new air speed. This usually required an interval of from 30 to 40 minutes. The experimental leaf was subjected to the light of three 100-watt lamps placed at a distance of about 3 ft. from the leaf chamber.

(a) Measurement of transpiration rate from the two surfaces of the intact leaf

After setting the leaf in the leaf chamber, each surface of the leaf was subjected in turn to half-hour periods of moving air in which the air was drawn

TABLE I

Date: 18.10.49. Total leaf area = 19.45 cm.^2

Exposed leaf areas: upper surface = 9.55 cm.^2 , lower surface = 9.35 cm.^2

Time: 10.30 a.m.—5.30 p.m.

Temperature: 16.0° C. – 17.5° C. Relative humidity: 94–92%.

Air speed (litres/hr.)	Upper surface		Lower surface	
	Time (min.)	Transpiration rate ($\text{mg. hr.}^{-1} \text{ dm.}^{-2}$)	Time (min.)	Transpiration rate ($\text{mg. hr.}^{-1} \text{ dm.}^{-2}$)
5	5	46.92	5	47.07
	10	46.92	10	47.07
	15	33.52	15	40.33
	20	40.22	20	40.33
	25	40.22	25	40.33
	30	40.22	30	40.33
10	5	58.12	5	46.62
	10	58.12	10	46.62
	15	58.12	15	46.62
	20	46.49	20	46.62
	25	46.49	25	46.62
	30	46.49	30	46.62
15	5	62.66	5	47.13
	10	62.66	10	47.13
	15	47.00	15	47.13
	20	47.00	20	47.13
	25	47.00	25	47.13
	30	47.00	30	47.13
20	5	55.29	5	55.44
	10	55.29	10	55.44
	15	55.29	15	55.44
	20	55.29	20	55.44
	25	55.29	25	55.44
	30	55.29	30	55.44

through the leaf chamber at speeds of 5, 10, 15, and 20 litres per hour. The results of a typical experiment are given in Table I and represented graphically in Fig. 1.

Examination of Fig 1 shows that in each experiment the amount of water-vapour carried over by the air stream fluctuates at first and comes to a constant rate after 15 to 20 minutes. The observed fluctuation is probably due to the

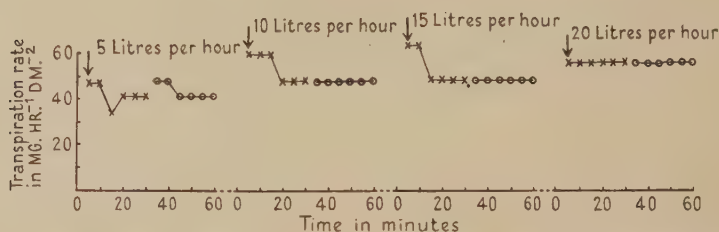


FIG. 1. Results of Expt. (a) (see p. 323) showing transpiration rates from intact lower surface (—○—) and from intact upper surface (—×—) plotted against time. The interruptions on the time axis represent intervals of 30-40 minutes.

time required for the thermocouple, evaporating surface, and humidity inside the leaf chamber to attain a steady state. The constant values of the transpiration rates attained after the first 15-20 minutes in each experiment are used for comparison. It may be observed that these transpiration rates are more or less equal from the two surfaces which, as already mentioned, have similar numbers of stomata (Table I and Fig. 1). The transpiration rates increase regularly with the increasing air speed (Fig. 1). In Table II, for convenience of comparison with what follows below, the actual rates are expressed as percentages of their maximal values at the highest air speed used.

TABLE II

Air speed (litres/hr.)	Upper surface		Lower surface	
	Actual rate (mg. hr. ⁻¹ dm. ⁻²)	Percentage of maximum	Actual rate (mg. hr. ⁻¹ dm. ⁻²)	Percentage of maximum
5	40.22	72.74	40.33	72.74
10	46.49	84.09	46.62	84.10
15	47.00	85.02	47.13	85.02
20	55.29	100.00	55.44	100.00

(b) *Measurement of evaporation from the exposed mesophyll as compared with the transpiration rate from the lower surface of one and the same leaf*

The leaf used for the last experiment was stripped of its upper epidermis, the lower epidermis being left intact. The leaf was set up in the leaf chamber in such a way that the areas of the evaporating surfaces were the same as in the previous experiment. The two surfaces were then subjected in turn to half-hour periods of moving air at air speeds of 20, 15, 10, and 5 litres per hour. The results are given in Table III and represented graphically in Fig. 2.

TABLE III

Date: 19.18.49.

Time: 1.30 p.m.—7.30 p.m.

Temperature: 17.5° C.—19.5° C. Relative humidity: 90–86%.

Air speed (litres/hr.)	Lower surface (intact)		Upper surface (stripped)	
	Time (min.)	Transpiration rate (mg. hr. ⁻¹ dm. ⁻²)	Time (min.)	Transpiration rate (mg. hr. ⁻¹ dm. ⁻²)
20	5	240.2	5	773.9
	10	166.3	10	829.2
	15	129.3	15	847.6
	20	166.3	20	847.6
	25	166.3	25	847.6
	30	166.3	30	847.6
15	5	125.7	5	532.6
	10	110.0	10	610.9
	15	110.0	15	626.6
	20	110.0	20	673.6
	25	110.0	25	673.6
	30	110.0	30	673.6
10	5	104.9	5	267.3
	10	69.93	10	313.8
	15	58.28	15	348.6
	20	46.62	20	360.3
	25	46.62	25	360.3
	30	46.62	30	371.8
5	5	67.22	5	127.4
	10	73.94	10	154.1
	15	67.22	15	180.9
	20	67.22	20	180.9
	25	60.49	25	194.4
	30	60.49	30	194.4
	35	60.49	35	194.4

Examination of Fig. 2 shows the results already mentioned under (a), namely, the increase of the evaporation rate with increase of the air speed. This increase is, however, much more pronounced for the stripped surface

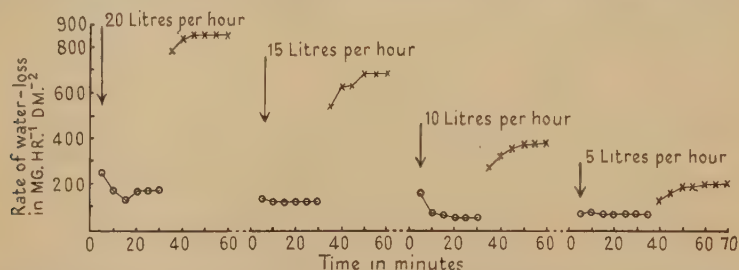


FIG. 2. Results of Expt. (b) (see p. 324) showing rates of water-loss from the intact lower surface (—o—) and from the stripped upper surface (—x—) plotted against time. The interruptions on the time axis represent intervals of 30–40 minutes.

than for the intact surface. In Table IV, as in Table II, the actual transpiration rates are expressed as percentages of their maximal values at the highest air speed used.

In the last column of Table IV, the excess of the rate from the stripped upper surface over the rate from the intact lower surface is expressed as a percentage of the rate from the intact surface. The high percentages in the last three cases are unexpected results.

TABLE IV

Air speed (litres/hr.)	Upper surface (stripped)		Lower surface (intact)		% increase on stripping
	Actual rate (mg. hr. ⁻¹ dm. ⁻²)	Percentage of maximum	Actual rate (mg. hr. ⁻¹ dm. ⁻²)	Percentage of maximum	
5	194.4	22.49	60.49	36.37	222
10	371.8	43.86	46.62	28.03	697
15	673.6	79.47	110.0	66.15	512
20	847.6	100.00	166.3	100.00	410

The rates of water-loss from the stripped upper surface were next compared with the transpiration rates from the same surface before stripping. The percentage increases due to stripping are given for different air speeds in Table V.

TABLE V

Air speed (litres/hr.)	% increase on stripping
5	125.6
10	399.9
15	549.9
20	713.1

In Table V it may be observed that the percentage increases are high and increase with the air speed.

To compare the rates of water-loss from the *lower surface* alone before and after removing the *upper epidermis*, the evaporation rates from the *lower surface* are next given for different air speeds as percentage increases on stripping the other surface (Table VI).

TABLE VI

Air speed (litres/hr.)	% increase on stripping
5	49.99
10	100.00
15	133.50
20	200.00

It will be observed from Table VI that the important and rather unexpected result emerges that on stripping the upper epidermis the rate of water-loss from the intact lower surface of the leaf may be increased according to the air speed by from 50 to 200 per cent.

Many similar experiments, which will be described more fully in a later communication, have yielded similar results confirming the above observations.

DISCUSSION

The increase of transpiration rate with increase of air speed both with intact and stripped leaves is evidently to be explained in the usual way as due to the increase in vapour-pressure gradient resulting from the layer of minimum vapour pressure approaching more closely to the leaf in moving air. Transpiration has been shown to increase with wind velocity by many workers, e.g. Knight (1917), Wrenger (1935), and Martin and Clements (1935). The diffusion resistance of the layer of still air surrounding the leaf (Bachmann, 1932; Welten, 1933; and Van den Honert, 1948) is reduced when this layer is partially removed.

The high percentage differences between the water-loss from the stripped and intact leaves given in the foregoing data are difficult to explain on current views of the low value of the resistance of open stomata.¹ These differences might be explained by assuming that the resistance of the stomatal system is actually much greater than is now generally supposed. The diffusive capacity of the stomatal system has been computed from that of multiperforate septa. Weishaupt (1935) has shown that pores 0.3 mm. in diameter with an aggregate area of 3.38 per cent. of the area of the septum allow 62 per cent. of the diffusion from an open evaporating surface. Huber (1930) has shown, too, that with pores of 0.05 mm. in diameter occupying 3.2 per cent. of the area of the septum the diffusion may be 72 per cent. of that from an open exposed surface. It is thus to be expected that the diffusive capacities of stomatal systems with open stomata might approach that of a free evaporating surface. Nevertheless, the present experiment would appear to indicate that the stomatal system constitutes a very considerable resistance to the diffusion of water-vapour from the mesophyll cells. Further experiments on these lines have amply confirmed this suggestion.

On the basis of these results it appears that the stomatal system constitutes a resistance sufficient to reduce the rate of water-loss at least to one-fifth. The only alternative explanations which suggest themselves are:

The stripping of the epidermis might affect the properties of the remaining mesophyll:

- (a) On stripping, the evaporating surface comes to consist of the torn middle lamella. Pectic substances thus come to constitute the immediate surface instead of cellulose, and the remaining half-wall will possess an asymmetrical constitution (cf. Hurst, 1948) instead of a symmetrical one like the original intact wall.
- (b) It is conceivable, also, that the water relations of the cell might be affected by the free access of atmospheric air to the exposed mesophyll.

It is proposed to examine these possibilities.

¹ That the stomata were in fact open was shown by the injection method. Xylol and benzene readily penetrated.

SUMMARY

Stripping off the epidermis of *Sedum* leaves is found to increase the rate of water-loss by amounts of the order of 700 per cent. A high resistance of the pores of the stomatal epidermis to the diffusion of water-vapour from the mesophyll is thus indicated.

Stripping one surface of a leaf considerably increases the rate of transpiration from the other (untouched) surface. The explanation of this is under investigation.

In conclusion, the writer wishes to thank Dr. F. M. Haines for suggestions, interest and criticism. Thanks are also due to the staff of the Chelsea Physic Garden and to Mr. I. R. McGregor for his valuable technical assistance.

LITERATURE CITED

- BACHMANN, F. (1932). Gedanken zur Transpirationsanalyse. *Planta*, **16**, 526.
 DARWIN, F. (1914). The effect of light on the transpiration of leaves. *Proc. Roy. Soc. B*, **87**, 281.
 GLOVER, J. (1941). A method for the continuous measurement of transpiration of single leaves under natural conditions. *Ann. Bot.*, N.S. **5**, 17, 25.
 HENDERSON, F. Y. (1926). On the effect of light and other conditions upon the rate of water-loss from the mesophyll. *Ibid.* **40**, 159, 507.
 HUBER, B. (1930). Untersuchungen über die Gesetze der Porenverdunstung. *Zeit. f. Bot.* **23**, 839.
 HURST, H. (1948). Asymmetrical behaviour of insect cuticle. *Discussion of the Faraday Society* ('The interaction of water and porous materials'), **3**, 193.
 KNIGHT, R. C. (1917). Recent work on transpiration. *New Phytol.* **16**, 127.
 — The interrelation of stomatal aperture, leaf water-content, and transpiration. *Ann. Bot.* **31**, 122, 221.
 LEWIS, F. J. (1948). Water movement in leaves. *Discussion of the Faraday Society* ('The interaction of water and porous materials'), **3**, 159.
 MARTIN, E. V., and CLEMENTS, F. E. (1935). Studies of the effect of artificial wind on growth and transpiration in *Helianthus annuus*. *Plant Physiol.* **10**, 613.
 VAN DEN HONERT, T. H. (1948). Water transport as a catenary process. *Discussion of the Faraday Society* ('The interaction of water and porous materials'), **3**, 146.
 WEISHAUP, C. G. (1935). *Diffusion of water-vapour through multiperforate septa*. Dissertation Ph.D., Ohio State University. (Quoted by Meyer and Anderson, *Plant Physiology*, D. van Nostrand Co. Inc., 1939.)
 WELTEN, M. (1933). Physiologisch-ökologische Untersuchungen über den Wasserhaushalt der Pflanzen mit besonderer Berücksichtigung der Wasserabgabewiderstände. *Planta*, **20**, 45.
 WRENGER, M. (1935). Über den Einfluss des Windes auf die Transpiration der Pflanzen. *Zeit. f. Bot.* **29**, 275.

Factors Controlling Flowering of the Chrysanthemum

I. THE EFFECTS OF PHOTOPERIOD AND TEMPORARY CHILLING

W. W. SCHWABE

*Research Institute of Plant Physiology, Imperial College of Science and Technology,
London*

WITH 2 PLATES

INTRODUCTION

QUITE early in the history of photoperiodism the Chrysanthemum was classified by Garner and Allard (1923) as a typical short-day plant. Since then it has been employed in experiments on photoperiodism by several investigators (Čajlachjan, 1936 and 1937; Moshkov, 1935 and 1937), who demonstrated that the length of day is perceived by the leaves. Using the Chrysanthemum they also showed that the stimulus can be translocated to the main shoot and other branches of the plant. Earlier, in America, Post (1931) and Poesch (1932) had also published work on the responses to day-length of the Chrysanthemum. Post (1939 and 1942) and Roberts and Struckmeyer (1938) investigated the effect of high and low temperatures on the flowering response of the Chrysanthemum. Both investigations led to the conclusion that in addition to a short photoperiod high temperatures are required for the rapid flowering of this plant. Post (loc. cit.) lays special stress on night temperatures, which he claims should be kept above 60° F.

During preliminary experiments in this institute it was noted that the response of Chrysanthemum to day-length treatment was not as uniform as had been expected from a study of the literature. There was a considerable amount of 'blindness', i.e. failure to produce inflorescence buds in short day (8 hours), while many plants budded although kept in long day (artificial and natural) or even in continuous light. A series of experiments involving day-length induction and other pre-treatments failed to elucidate the cause of this behaviour. The only clear-cut difference due to day-length was an effect on the further development of the 'potential inflorescence' buds produced. In long-day conditions these failed to enlarge and open, while lateral vegetative shoots grew out of the axils of leaves directly below the bud in place of the subsidiary inflorescences which are normally produced in short day. This phenomenon is usually described as 'natural break-bud formation'; it has occurred in all varieties tested here. The potential inflorescence buds, thus formed, usually die if maintained in long day, but if transferred to short day will produce open flowers which are more or less normal according to the time spent in long day (Fig. 1). It was clear from these experiments, which need not be described

here in detail, that in the reaction of the *Chrysanthemum* to length of day two separate problems are involved: (1) that of inflorescence initiation, and (2) that of the further development of the potential inflorescence bud. This paper is concerned with the first aspect only. Taking all the original series of experiments together, a significant seasonal difference was noticed. Those experiments which were started in early summer or later in the season delayed their bud production until the following spring, while those started in the early spring initiated inflorescence buds in the same season. Also, large differences in behaviour appeared in two greenhouses kept at different temperatures. Thus in two experiments in 1947-8 the plants grown in a heated greenhouse (whose temperature was generally kept above 12.5°C.) required on the average 194 days before the macroscopic appearance of the inflorescence bud, while those in the unheated house required only 130 days. Similar but larger differences were found in the total numbers of leaves (excluding all bracts) produced by these plants. The number of leaves to the inflorescence bud may be taken as a relatively objective measure of the total vegetative growth of the plant over the experimental period before the reproductive phase begins (Purvis and Gregory, 1937); the leaf numbers noted were: heated house 112 leaves; temperate house 49.

This observation suggested that the temperature factor required to be reinvestigated, and that the need for a temporary exposure to cold (i.e. vernalization) might be the factor delaying inflorescence bud initiation. Hence a series of experiments was undertaken to explore the possible effects of vernalization.

METHODS

The *Chrysanthemum* is usually propagated by cuttings and it is therefore possible to work with a clone derived from a common parent plant. The use of such material entails the disadvantage that it is impossible to exclude entirely the possibility of a carry-over from a previous treatment, in particular day-length induction. In order to avoid this possibility two stock lines were maintained, one in continuous long-day and the other in short-day conditions. Hence, unless otherwise stated, all cuttings were of known day-length history; they were always taken from the base of the parent plant and at the time of rooting had not more than five leaves separated from the main bud, of which three were fully expanded. Rooting was usually complete after 2-3 weeks in a warm greenhouse.

Throughout the season all plants were maintained in a greenhouse whose temperature was kept at or above 15.6°C. The day-length treatment consisted of 8 hours' full daylight (9 a.m. to 5 p.m.), after which the plants were transferred to special dark chambers for the night. Long-day treatments received a further $8\frac{1}{2}$ hours of low-intensity illumination given by incandescent lamps suspended over the plants; these provided about 25 ft. candles at plant height. Only in a few experiments were the plants given full daylight for more than 8 hours.

The vernalization treatment was usually given by placing the rooted cuttings in a refrigerator divided into two light-tight compartments, so that two day-length treatments (8 and 16 hours) could be maintained, the light intensity being about 25 ft. candles. Owing to the heat produced by the incandescent lamps the temperature during the light phase in the refrigerator tended to rise a few degrees above the level prevailing during the dark period. Average maximum and minimum temperatures during one experiment were: min. 0.94°C. ; max. 7.4°C. Though older plants may be successfully vernalized, the usual practice consisted in giving the chilling treatment as soon as the cuttings had rooted in order to obtain the maximum effect. The plants were rooted and vernalized in flats, later they were grown on singly in square tins holding approximately 12 lb. of soil. They were not stopped (pinched). In a few cases where the main shoot was broken accidentally a single lateral was allowed to grow on.

The data collected and presented below are: (a) number of leaves produced to the inflorescence (cf. above), and (b) the time required for the production of the macroscopically visible inflorescence bud.

RESULTS

1. *The interaction of vernalization with length of day*

In a preliminary experiment started on 16 January 1948 slightly older cuttings were used, all of which had been raised in long day and before attention had been focused on the importance of temperature control. Some were kept at high temperature throughout the experiment while others were given 1 or 2 months of cold treatment by placing them out of doors, after which they were returned to the greenhouse. Each treatment was replicated ten times. All plants received short day throughout the experiment. Mean maximum and minimum temperatures for these periods were as follows: first month, max. 8.3°C. , min. 2.6°C. ; second month, max. 10.1°C. , min. 1.1°C. The results of the experiment are shown in Table I.

The plants from all cold treatments show significantly reduced leaf numbers, but only the treatment of 1 month's cold at the start induced earlier flowering than in the controls. This time, of course, includes the period of chilling during which the growth of the plants was nil. Comparison should therefore be confined to the time to budding after the completion of the cold treatment. These times are shown in the next column and represent the real difference between those plants receiving vernalization treatments and their high temperature controls. It is also worthy of note how much more uniform the flowering behaviour had become as a result of chilling (spread in days between inflorescence bud production in the earliest and latest plant).

Following this preliminary investigation, two factorial experiments were set up combining vernalization and day-length. In the first experiment freshly rooted 5-leaf cuttings were used, 16 per treatment. Half of these had been taken from long-day and half from short-day parents; however, in the tabulation

TABLE I

Vernalization by outdoor temperatures

Experiment started 16 January 1948. Variety 'Indian Summer'. Short day (11 hrs.) throughout. Warm greenhouse ($> 15.5^{\circ}\text{C.}$). Ten replicate plants.

Pre-treatment	Leaf number to inflorescence	Days to flowering		Spread in days between budding of first and last plant
		After start of expt.	After return to high temperature	
High temperature throughout	54.1	94	—	58 days
2 months at low temperature	40.6	94	37.5	5 "
1 month at low temperature	42.4	79	47.8	5 "
1 month at high temperature followed by 1 month at low temperature	42.8	94	37.9	19 "
Significant difference	6.2 leaves	7.5 days		

of the results, these two sets have been pooled. Again leaf numbers and times to flowering have been recorded and are shown for two dates. Table II was compiled on 25 January 1949, i.e. 36 weeks after the start of the experiment and almost 6 months after all vernalized plants in short day had become fully budded. The plants vegetative on that date were kept on for another 4 months and the flowering times and leaf numbers of these are given in Table III.

TABLE II

Interaction of vernalization and length of day

Experiment started 18 May 1948. Variety 'Indian Summer'. Warm greenhouse ($> 15.5^{\circ}\text{C.}$). 16 replicate plants. Date of observation, 25 January 1949.

	Short day (8 hrs.)				Long day (16½ hrs.)			
	Number of plants budded	Leaf number to inflorescence	Days to flowering		Number of plants budded	Leaf number to inflorescence	Days to flowering	
			From start	After return to greenhouse			From start	After return to greenhouse
Vernalized	16	28.6 ± 1.8	81 ± 2.7	39 ± 2.7	9	44.1 ± 4.7	148 ± 16.8	106 ± 16.8
Unvernalized	4	61.8 ± 13.5	112 ± 21.2	—	2	49.5 ± 13.5	141 ± 33.5	—

TABLE III

Plants showing delayed budding or remaining vegetative

Date of observation, 17 May 1949.

	Short day					Long day	
	Number of plants budded after 36 weeks	Number remaining vegetative after 1 year	Leaf number		Days to flowering	Number remaining vegetative after 1 year	Leaf number of vegetative plants
			To inflorescence	Of vegetative plants			
Vernalized	—	—	—	—	—	7	64.1 ± 1.9
Unvernalized	9	3	135.0 ± 4.5	147.3 ± 10.5	351.7 ± 6.4	14	83.6 ± 4.1

Vernalization and short day are again seen to cause rapid flowering with very low leaf numbers. If allowance is made for the fact that the plants had differentiated about 14 leaves by the time the cold treatment was begun (determined by dissection of some replicate plants), the increment in leaf number before inflorescence bud initiation is as low as 14, and the number of days after vernalization to the macroscopic appearance of the inflorescence bud as low as 39. Of the long day vernalized set 9 out of 16 had budded with rather higher leaf numbers and after a much longer time. Only four plants of the high temperature controls in short day had flowered by this time with terminal inflorescences, but two more had produced small flowers in lateral axils some distance below the main apex. They did so with leaf numbers more than twice as large as those of the vernalized set. Only two unvernallized plants in long day formed potential inflorescence buds.

All plants budded after 25 January 1949 were from the unvernallized short-day treatment. Their leaf number was more than 4 times as large as the vernalized set, while the time required for their buds to be formed was almost 9 times as long. The remaining long-day plants stayed vegetative. Including all sixteen replicates in each of the four treatments, the final leaf numbers produced on the main axis, by the end of the experiment, were: vernalized short day 28.6, vernalized long day 52.9; unvernallized short day 114.7, unvernallized long day 79.3.

Internode length was also very much increased by vernalization. The differences between the two short-day sets were very great (see Fig. 2) and even in the two long-day groups there was a highly significant increase due to vernalization; the mean internode lengths were: for vernalized long day 2.58 cm.; unvernallized long day 1.91 cm., with significant difference 0.26 cm.

One of the most striking effects noted in this experiment was the diageotropic growth habit exhibited by all the high temperature short-day plants (see Figs. 2 and 3). This type of growth occurs normally in the underground stolons of the Chrysanthemum. It was evident at a very early stage, soon after the cuttings commenced to grow vigorously. Short-day cuttings from short-day parents at high temperature were the first to assume the habit. One or two unvernallized cuttings in long day also showed signs of it initially but later turned upwards again. Those unvernallized short-day plants which flowered after a long delay also became erect again at this stage. This type of growth is reported to have been observed in Chrysanthemums grown in tropical conditions.

The second confirmatory experiment using the same variety was carried out in 1948-9. In this experiment only one plant of the unvernallized series budded in short day and then only after reaching a very high leaf number. All vernalized plants budded *regardless of day-length*. One of the vernalized short-day plants behaved somewhat aberrantly by budding much later than the other fifteen replicates; in Table IV the results of this treatment are shown including this plant and also, in brackets, excluding it. All unvernallized plants in short day again showed transverse geotropism, and the results are even more clear-cut than in the previous experiment.

TABLE IV

Interaction of vernalization and length of day

Experiment started 13 December 1948; ended after 273 days. Variety 'Indian Summer'. Mean maximum and minimum temperatures during 6 weeks' vernalization: max. 7.4°C ., min. 0.9°C .

	Number of replicates	Number of plants budded	Leaf number to inflorescence	Leaf number of vegetative plants after 9 months	Days to flowering	
From start					After return to greenhouse	
Vernalized						
Short day .	16	16	27.8±3.0	— **	118.1±4.7	76.1±4.7
	(15)	(15)	(24.8±0.56)	—	(113.5±1.5)	(71.5±1.5)
Long day .	11	11	29.1±2.5	—	137.7±8.8	95.7±8.8
Unvernalized						
Short day .	16	1	98	102.1±1.6	219	—
Long day .	11	0	—	75.2±3.5	—	—

The results of a first experiment carried out to assess the vernalization effect quantitatively are shown in Fig. 4. Cuttings from long- and short-day parents were vernalized and later maintained in their original day-lengths. Vernalization was given for periods increasing from 1 to 9 weeks by steps of 1 week. Owing to lack of greenhouse space, only three replicates of each treatment could be grown.

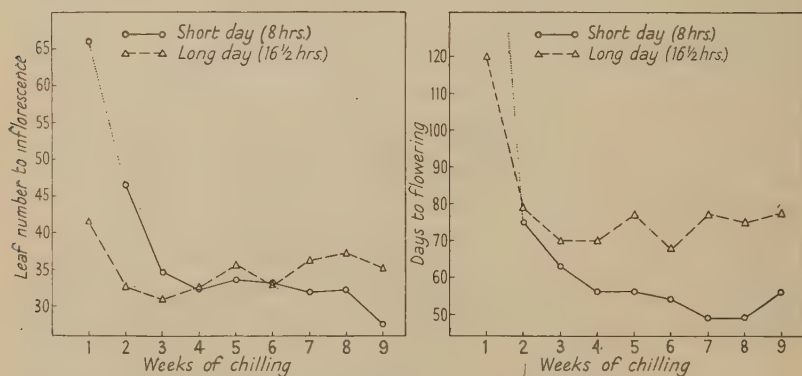


FIG. 4. Effect of duration of vernalization treatment on leaf number and time to macroscopic appearance of inflorescence bud. Since only one replicate flowered in short day after 1 week's vernalization, the slope indicated by the dotted line is the minimum possible, i.e. if all plants had flowered at the end of the experiment with the leaf number present at that time.

It is clear from the leaf-number curves as well as from those for time to flowering that as short a period of low temperature as 3 weeks is sufficient for the attainment of the full vernalization effect. Even a fortnight's chilling appears to be sufficient to cause all plants to flower either in short or long day; in the 16-hour day leaf numbers are already minimal but the time to flowering

is capable of further reduction. In short day, however, both characteristics are much above the minimum. After a single week's chilling only one short-day plant budded and that with quite a high leaf number; the other two replicates remained unvernallized, exhibiting diageotropic growth. In long day, on the other hand, all three replicates formed potential inflorescence buds, but again only after a very long time and with high leaf numbers. Clearly, therefore, in the variety tested the vernalization effect is predominant over day-length effects in so far as inflorescence bud initiation is concerned. Unfavourable day-lengths on the whole prolong the period of vegetative growth, though to a lesser extent than does absence of vernalization in favourable day-lengths.

2. Discontinuous vernalization during the light and dark phases

Two experiments were set up designed to show whether the cold treatment needs to be continuous to be effective, and if not whether a difference in the effectiveness of the cold treatment exists as between application during the light and during the dark phase. The plants were vernalized by placing them out of doors during the cold season, as it was impracticable to produce illumination in the refrigerator comparable in brightness to that in the greenhouse, even in winter conditions.

In the first experiment carried out with plants from long day and of unknown temperature history the treatments were given continuously from 20 January until the plants had budded. They received short photoperiod (11 hrs.) throughout. The four treatments included two sets of alternating high and low temperatures in the light and dark phases, as well as controls in continuous high and continuous low temperatures.

The results are shown in Table V. The mean maximum and minimum temperatures for day and night 4 ft. above ground were calculated for 20 weeks after the start of the experiment:

TABLE V

Discontinuous vernalization during the light and dark phases

Experiment started 20 January 1948. Variety 'Indian Summer'. Short day (11 hrs.) throughout. 6 replicate plants

Outdoor temperatures: day		max. 11.7° C.	Min. 5.9° C.	Spread in days between first and last plant budding
night		„ 9.0	„ 4.9	
Temperature treatment		Leaf number to inflorescence	Days to flowering from start	
During light	During dark			
High	High	61.2	127	57 days
High	Low	46.3	140	8 „
Low	High	>79.5	>209	>177 „
Low	Low	43.2	153	9 „
Significant difference		14.1	36	—

Leaf numbers were significantly reduced by continuous cold treatment, but also by cold nights alone. It appears that flowering behaviour was much

had dark green foliage with large amounts of a red pigment—probably anthocyanin—while those chilled only in day-time showed severe chlorotic symptoms in the young leaves which quickly disappeared on return to warm days. This effect of low day and high night temperatures has also been described by Roberts and Struckmeyer (1938).

3. Need for annual re-vernalization

Basal shoots, such as were used in the above experiments, are normally produced on plants soon after their flowering period is ended. It is these shoots which are normally used as cuttings in horticultural practice and it seemed important to discover whether they need to be re-vernalized or whether there is a carry-over from the previous season's shoots. The experiment, summarized in Table VII, was therefore set up using cuttings from previously vernalized plants which had flowered in short day. These cuttings were taken after the main shoots had started to die off, a stage when the plant normally enters a state of dormancy.

TABLE VII

Re-vernalization of cuttings from vernalized plants having flowered in short day

Experiment started 12 November 1948, ended 12 September 1949. Variety 'Indian Summer'. Short day (8 hrs.) throughout.

Temperature treatment	Number of replicates	Number of plants budded	Leaf number to inflorescence	Days to flowering	
				From start	After return to greenhouse
Vernalized	20	20	33.6 ± 1.1	118.3 ± 1.6	76.3 ± 1.6
Unvernalized	23	17	72.0 ± 4.7	187.1 ± 8.7	—
		[vegetative 6]	97.3 ± 2.2	—	—

The results show clearly that any induction effect on the young basal shoots produced on plants flowered in short day is at best only very slight and they require to be vernalized afresh, hence under ordinary conditions vernalization is needed annually.

4. Varietal differences

Apart from the variety 'Indian Summer' used for the main experiments, five other varieties of *Chrysanthemum* have been grown and four of them tested for their response to vernalization treatment. The fifth variety 'Perennial White' was not tested as this variety cannot be prevented from forming buds, some of which produce open flowers, even by the combination of high temperature and long day. All the other varieties responded to the vernalization treatment in the usual manner by reduced leaf numbers and accelerated bud formation; but as there was some doubt about the temperature history of some of the cuttings, it is uncertain whether the maximum vernalization effect was obtained.

There are, however, certain residual differences which must be ascribed to varietal effects. For instance, the single variety 'Godfrey's Gem' resembles the 'Perpetual White' cited above in that it cannot be entirely prevented from budding by long-day treatment in the absence of vernalization. Nevertheless, considerable differences in leaf number and time to inflorescence bud initiation are found between the vernalized and unvernallized treatments.

Transverse growth in conditions of high temperature and short day was found in all but one variety. This variety, 'Cossack', tended to produce short stems of abnormally large diameter. In the variety 'Poitevine' fasciated shoots have been found under these conditions.

An experiment similar to those reported in section 1 was carried out using the horticultural species *Chrysanthemum rubellum*¹ (Sealy, 1938), in order to test a quite different Chrysanthemum plant. The results were precisely similar to those for *C. morifolium*, even to the extent of diageotropic growth in the short-day unvernallized set, though the onset of this habit was considerably later than in the other species.

Again all unvernallized plants failed to flower, though the experiment was carried on for 28 weeks. Two of the vernalized plants kept in short day clearly became de-vernalized after some time. Quite suddenly the length attained by their new internodes was shortened to the same extent as in the unvernallized plants and their stems also began to grow horizontally. Further vernalization for 2 weeks, however, was sufficient to bring these plants into flower (Fig. 5). In Table VIII the data from the short-day vernalized plants are shown both *in toto* and omitting these two plants (brackets). As *C. rubellum* has a somewhat different growth habit leaf numbers refer to those expanded after the start of the experiment, omitting those present at the time of taking the cuttings.

TABLE VIII

Interaction of vernalization and length of day in Chrysanthemum rubellum

Experiment started 10 November 1948, ended 26 May 1949. Vernalization treatment 26 days.

	Number of replicates	Number of plants budded	Leaf number to inflorescence	Leaf number of vegetative plants after 28 weeks	Days to flowering	
					From start	After return to greenhouse
Vernalized						
Short day	6 (4)	6 (4)	33.3 ± 7.2 (22.0 ± 0.71)	—	111.8 ± 20.6 (79.3 ± 1.3)	85.8 ± 20.6 (53.2 ± 1.3)
Long day	6	6	31.3 ± 4.1	—	86.0 ± 5.8	60.0 ± 5.8
Unvernallized						
Short day	6	—	—	74.5 ± 4.6	—	—
Long day	6	—	—	80.8 ± 1.4	—	—

Compared with the large effects of vernalization in reducing leaf number, &c., day-length effects on flower-bud initiation are negligible. However, the further development and opening of the buds were inhibited in long day.

¹ I am indebted to the late Dr. Alex Smith for the kind gift of this species.

DISCUSSION

The results established by the above experiments may be summarized as follows: It is clear that a period of low temperature, i.e. vernalization, is necessary for (or at least greatly accelerates) the flowering of the Chrysanthemum plant. There is evidence that little or none of the effect of vernalization is normally carried over into the young basal shoots produced at the time when the old stem of the plant is beginning to die (Table VII). In a number of experiments Čajlachjan (1937) caused basal shoots to flower usually after defoliating them and decapitating the main shoot; in one or two cases he succeeded in getting the basal shoots to flower without these treatments, but, judging by his photograph, the basal shoots appear to have been produced early in the growth of the plant and are not truly comparable to the type of shoot referred to above; they must rather be regarded as low laterals of the main shoot.

The time required for complete vernalization is relatively short: about 3 weeks are sufficient to produce the full effect, longer cold treatment up to at least three times this period seeming to have no further effect. Regarding the temperature level, from the data presented here it is certain that ordinary outdoor temperatures from January to March, and 5–7° C. in the refrigerator, are fully effective. It is probable that somewhat higher temperatures may also stimulate flowering. However, in the absence of adequate temperature control it was not possible to assess these effects quantitatively.

Although the results are few, there is a strong indication that different varieties behave in fundamentally the same manner and require to be vernalized. However, there appear to be quantitative differences. Some varieties, such as 'Godfrey's Gem', it appears, cannot entirely be prevented from flowering by continued high temperature in long day or short day but always produce inflorescence buds after reaching high leaf numbers. The variety 'Perpetual White' is one from which day-length specificity and vernalization requirement have been almost completely eliminated. Though very few varieties were tested, there appears to be some correlation between the readiness to flower generally and the need for vernalization. Those varieties which are most easily prevented from flowering by continuous high temperature tend also to display greatly delayed budding in long days. A preliminary experiment involving the vernalization of young plants raised from seed also gave some indication of the hastening effect of vernalization.

At first sight it seems rather surprising that this need for vernalization treatment was apparently overlooked by horticulturists and others who have previously worked with this plant. However, the normal horticultural practice is to take cuttings early in the year (January–March, usually) and to keep the stock plants during the winter in greenhouses whose temperatures are maintained only a few degrees above freezing-point; it is clear therefore that the cold-temperature requirement may easily be satisfied by this practice. This is particularly likely since exposure to cold during the night alone is

fully adequate. Wherever details are presented it appears from the papers of Čajlachjan, Post, Roberts, and Struckmeyer, &c., that their cuttings or plants were obtained from horticultural sources and are thus likely to have received their vernalization prior to experimental treatment. Furthermore, in nearly all the experiments of the above authors the plants were also given a period of long day before being subjected to other treatments. Although preliminary experiments here make it appear somewhat unlikely, the possibility still exists that long day pre-treatment may to some extent act as a substitute for vernalization, in an analogous manner to the effect of short day on winter-rye (Purvis and Gregory, 1937). Further work in this direction is now in progress. If this possibility should prove correct it would provide a further explanation of the fact that the need for vernalization has not been noticed in the past. This applies also to the experiments reported by Post in 1939. His temperature treatments were given to plants at an advanced stage of development. The temperature history of the plants is not stated, but they appear to have been raised in the usual horticultural fashion and were given extensive periods of long day prior to the experimental treatment. It is probably significant, however, that the author found that late propagation increased the amount of blind growth and that 'shoots which failed to form flower buds during the early fall at high temperatures remained blind during the winter even though the temperature was increased'.

The inhibiting effects of low temperature noted by Post and Roberts and Struckmeyer can probably be ascribed largely to a lowered growth rate, for their plants were kept permanently in this condition. In the present experiments cuttings exposed to the cold in the refrigerator ceased to grow altogether, and Roberts and Struckmeyer actually state that in their low-temperature plants also there was practically no increases in size. It seems probable that Post (1947*a*) may actually have encountered the problem of vernalization. In his paper entitled 'Year round Chrysanthemum production' he mentions difficulties in the production of flowers on early varieties from February to July and on late varieties from May to August. This suggests the possibility that the plants used may have been grown from summer cuttings, which had not been vernalized at all or at least not adequately. In another paper (1947*b*) he refers to '... hardy varieties that never flowered ...' and rejects high temperatures in August and September as the possible cause. Again lack of vernalization may be suspected.

A further point in earlier work requiring explanation is the failure to note the formation of potential inflorescence buds, or failure to regard plants producing them as *flowering*. Hamner (1938), for instance, states: 'Under similar conditions of photoperiods greater than the critical the flower buds of Chrysanthemum may abort completely and the plant as a whole be definitely vegetative.' Initially Post (1934) seems to have entertained some doubts as regards the character of these buds, for he states: 'It is entirely possible that there is no difference between the two at their time of formation, but the crown bud is nothing more than a terminal bud differentiated under short

day conditions and developed to some extent under long day conditions.' Adhering to the idea of an absolute dependence of the Chrysanthemum on short day for its flower formation, he supposes that cloudy mornings and evenings have shortened the natural long day sufficiently to result in short-day induction. However, considering the low light intensities effective in prolonging the photoperiod artificially, this explanation seems hardly probable. Later (1947*b*) Post actually states: 'It is much easier to delay the development of flower buds with artificial light than to prevent their formation'; and this description of the effect of long day whether artificial or natural is in full agreement with the present experimental results. Behrens (1949), on the other hand, clearly recognizes that Chrysanthemum will form inflorescence buds in continuous long day, though after a considerable delay. Observation of outdoor Chrysanthemums demonstrates that the production of potential inflorescence buds during the long days of summer is the rule rather than the exception and has been found to occur in nearly all varieties—the exact time depending to some extent on the earliness of the particular variety.

Apart from the Chrysanthemum only one other short-day plant has been recognized as requiring vernalization. As Hamner stated in 1944, all plants known to be vernalizable are favoured by long day. However, Peterson and Loomis (1949) have found a rather similar situation in the Kentucky Blue Grass, confirming some earlier observations by Roberts and Struckmeyer (1938). This grass seems to require a temporary exposure to low temperature during the winter for flower initiation, but its dependence on short day appears to be much more rigid than in the Chrysanthemum.

One further aspect needs to be considered. It concerns the probable relation of the auxin metabolism of the Chrysanthemum to vernalization effects. That there are relations is indicated by the large effects on the elongation of internodes and in particular by the diageotropic growth habit exhibited by the short-day plants in an unvernallized condition. The effect on internode length appears to be analogous to the shooting in cereals and elongation of the main axis of such biennials as *Hyoscyamus niger*. Hence it seems highly probable that the plant's auxin metabolism is modified by vernalization. Increase in internode length suggests a quantitative change either in auxin content or possibly in the sensitivity of the tissues to its action. Changes in the geotropic reaction suggest differences in the distribution. It would seem unlikely that these changes should be unrelated to the flowering behaviour itself, though there is no means of distinguishing between cause and effect on the basis of the present experiments.

A number of papers have appeared recently which tend to show that auxin exerts an inhibiting influence on flowering (ThurLOW and Bonner, 1947; Green and Fuller, 1948; Bonner and ThurLOW, 1949). Leopold and Thimann (1949), however, come to the conclusion that there is an optimum auxin level for flower formation in the plant. Judging solely from morphological characters, such as internode length and apical dominance, it is not clear how the present experiments can be fitted into such a scheme, for, while it may be assumed

that the short-day unvernallized plants (vegetative) are below the minimum auxin level for flowering (very short internodes and somewhat reduced apical dominance), the non-flowering long-day unvernallized set must be placed near to or above the short-day vernallized treatment (optimal for flowering) on account of only slightly shorter internode lengths but very strong apical dominance, finally the longest internodes are found in the long-day vernallized set which also initiate inflorescence buds, but has a somewhat weakened apical dominance. It would be equally difficult—on the basis of internode length effects, &c.—to reconcile these experiments with a theory which regarded auxin as the sole agent controlling flowering on the basis of its inhibitory effect, for again the appearance of the short-day unvernallized plants suggests minimal auxin levels.

Although the facts presented in this paper could not be accounted for on a simple hypothesis of auxin control of flowering, it seems probable that changes in auxin metabolism result from low-temperature and photoperiodic treatments and some relation between flowering and auxin metabolism is suggested. Further work to elucidate these relations is projected.

SUMMARY

1. Experiments are described which indicate that a temporary exposure to low temperature (vernallization) hastens inflorescence bud initiation in the *Chrysanthemum*, as measured by the time to the macroscopic appearance of the bud and also by the number of leaves produced. This effect is found in both long- and short-day conditions.

2. In the absence of vernallization plants kept in short day assume a diageotropic growth habit and remain vegetative for long periods, frequently for much more than one year. Unvernallized long-day plants also remain vegetative but have a normal geotropic reaction.

3. While the day-length effect is less important for inflorescence bud initiation, the opening and further development of buds formed in long day depend normally on subsequent day-length treatment.

4. A vernallization period of only three weeks appears to be fully adequate.

5. The low-temperature treatment may be given discontinuously, and evidence to hand appears to indicate that it is more effective if given during the dark phase than during the light phase. Hence de-vernallization by temperatures of about 20–25° C. does not appear to take place.

6. There is evidence that little or none of the stimulus is carried over from one year to another.

7. The results are discussed in relation to the auxin metabolism of the plant and also with regard to the absence in the literature of previous mention of the cold requirement.

The author is indebted to Professor F. G. Gregory and to Mr. F. J. Richards for their stimulating interest and helpful suggestions in the course of this



FIG. 2

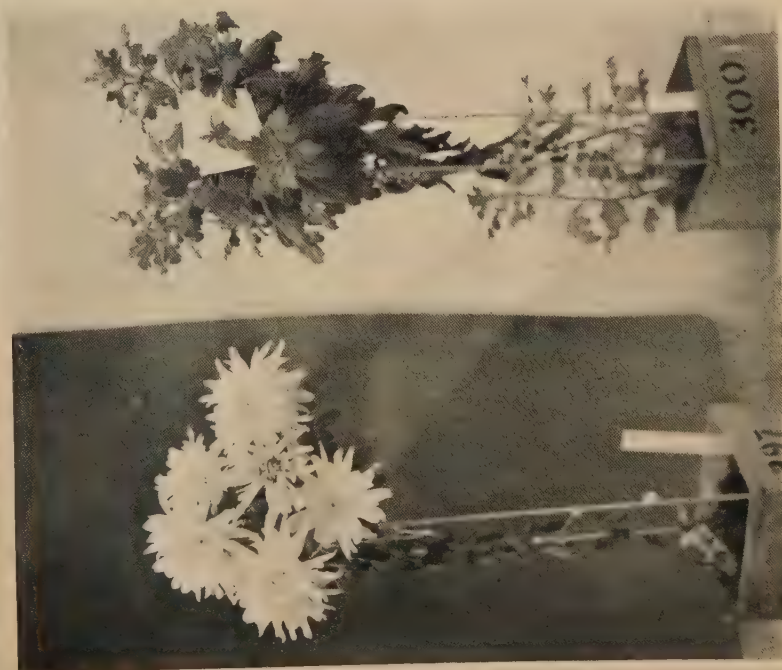


FIG. 1

W. W. SCHWABE—PLATE I



FIG. 3



FIG. 5

work, and to Dr. M. Holdsworth for providing him with the results of earlier unpublished work.

Messrs. H. Woolman Ltd. kindly supplied some of the plant material used.

LITERATURE CITED

- BEHRENS, G. (1949). Blüten- und Gestaltsbildung bei *Chrysanthemum* und *Sempervivum* unter photoperiodischen Einflüssen. *Biol. Zentralbl.* **68**, 1-32.
- BONNER, J., and THURLOW, J. (1949). Inhibition of photoperiodic induction in *Xanthium* by applied auxin. *Bot. Gaz.* **110**, 613-24.
- ČAJLACHJAN, M. H. (1936). On the mechanism of photoperiodic reaction. *Comptes Rendus (Doklady) de l'acad. sci. U.R.S.S.* **1**, 89-93.
- (1937). *Hormonal theory of plant development*. Publ. Acad. Sci. U.S.S.R. Moscow.
- GARNER, W. W., and ALLARD, H. A. (1923). Further studies in photoperiodism, the response of the plant to relative length of day and night. *J. Agr. Res.* **23**, 871-920.
- GREEN, M., and FULLER, H. J. (1948). Indole-3-acetic acid and flowering. *Science*, **108**, 415-16.
- HAMNER, K. C. (1938). Correlative effects of environmental factors—Photoperiodism. *Bot. Gaz.* **99**, 615-29.
- (1944). Photoperiodism in plants. *Ann. Rev. Biochem.* **13**, 575-90.
- LEOPOLD, A. C., and THIMANN, K. V. (1949). The effect of auxin on flower initiation. *Amer. J. Bot.* **36**, 342-7.
- MOSHKOV, B. S. (1935). *Bul. Appl. Bot., Genet. and Pl. Breed.* **17**, 25-30.
- (1937). *Ibid.* **21**, 145-56, as quoted by Chlodny (1939), *Herbage Rev.*
- PETERSON, M. L., and LOOMIS, W. E. (1949). Effects of photoperiod and temperature on growth and flowering of Kentucky bluegrass. *Plant Physiol.* **24**, 31-43.
- POESCH, G. H. (1932). Further studies of photoperiodism of *Chrysanthemum*. *Proc. Amer. Soc. Hort. Sci.* **29**, 540-3.
- POST, K. (1931). Reducing the daylength of *Chrysanthemums* for the production of early blooms by the use of black sateen cloth. *Ibid.* **28**, 382-8.
- (1934). Production of early blooms of *Chrysanthemum* by use of black cloth to reduce the length of day. *N.Y. (Cornell) Agr. Expt. Sta. Bull.* 594.
- (1939). The relationship of temperature to flower bud formation in *Chrysanthemum*. *Proc. Amer. Soc. Hort. Sci.* **37**, 1003-6.
- (1942). Effects of daylength and temperature on growth and flowering of some florist crops. *N.Y. (Cornell) Agr. Expt. Sta. Bull.* 787.
- (1947a). Year-round *Chrysanthemum* production. *Proc. Amer. Soc. Hort. Sci.* **49**, 417-19.
- (1947b). *Chrysanthemum* troubles of 1947. *New York State Flower Growers Bull.* **27**, 4-8.
- PURVIS, N., and GREGORY, F. G. (1937). Studies in the vernalization of cereals. I. A comparative study of vernalization of winter rye by low temperature and short days. *Ann. Bot.*, N.S. **1**, 569-92.
- ROBERTS, R. H., and STRUCKMEYER, B. E. (1938). The effects of temperature and other environmental factors upon the photoperiodic response of some of the higher plants. *J. Agr. Res.* **56**, 633-77.
- SEALY, J. R. (1938). *Chrysanthemum rubellum* and its allies. *J. Roy. Hort. Soc.* **63**, 265-73.
- THURLOW, J., and BONNER, J. (1947). Inhibition of photoperiodic induction in *Xanthium* by applied auxin. (Abstr.) *Amer. J. Bot.* **34**, 603.

DESCRIPTION OF PLATES

FIG. 1. Plants budded in long day. Left: plant transferred to short day. Right: plant maintained in long day (arrested bud at the centre of the white card).

FIG. 2. Interaction of day-length and vernalization in *C. morifolium* var. 'Indian Summer'. Left to right: Vernalized short day, vernalized long day, unvernallized long day, unvernallized short day.

FIG. 3. Unvernallized plant in short day showing diageotropic growth habit.

FIG. 5. Interaction of day-length and vernalization in *C. rubellum*. Left to right: Short day unvernallized, long day unvernallized; long day vernalized, short day vernalized.

The Crystalline-Non-Crystalline Ratio in Celluloses of Biological Interest

R. D. PRESTON

Department of Botany, Leeds University

AND

P. H. HERMANS AND A. WEIDINGER

Instituut voor Celluloseonderzoek, Utrecht, Holland

I. INTRODUCTION (by R. D. PRESTON)

OF late years a good deal of attention has been paid to the question of the relative amounts of crystalline and non-crystalline material in cellulose, but it has been an almost invariable feature that interest has centred around problems of cellulose technology, and the work has therefore been carried out with celluloses of commercial importance. Certainly these have included both cotton hairs and phloem fibres which are of immediate interest also in the botanical field, but in the lack of any organized attempt to examine these from a botanical standpoint they can hardly be said to constitute any serious contribution in a strict botanical sense. It has become abundantly clear through these researches, however, that the crystalline-non-crystalline ratio is of fundamental importance in assessing the qualities of a fibre, since some properties depend more on the non-crystalline fraction of the cellulose and others on the crystalline. It is quite certain, therefore, that the determination of this ratio will be of equal importance in cell-wall studies themselves, since the very physical properties of fibres which are exploited industrially are the same properties which must be considered in problems of cell expansion and growth generally. As yet it is much too early to point out any immediate value which might accrue from this particular point of view, but it might perhaps be said that if it turns out that the connexion between the protoplasmic surface and wall deposition is as close as it is now supposed in many quarters to be (Frey-Wyssling, 1948; Preston, 1949; Preston and Wardrop, 1949*a*), then work of this kind may prove vital to a complete understanding of the nature of the protoplasmic surface.

The present paper is the result of the application of a quantitative X-ray method for determining the percentage crystallinity in cellulose fibres, recently developed by P. H. Hermans and A. Weidinger, to some objects of botanical interest prepared in the laboratory of R. D. Preston. This investigation is not intended to convey any coherent study of any particular problem, but to present the results of the study of a rather heterogeneous collection of material sufficiently interesting botanically as to give a lead into the problems involved.

Perhaps before describing the material used a word might be said as to the meaning here of the terms *crystalline* and *non-crystalline* as applied to cellulosic material. The general conception is that within any chosen cellulosic body there exists, side by side, molecular chains of cellulose organized into strictly

regular crystalline space-lattices (roughly, therefore, into the classical 'micelles' of Nageli) and chains which are more randomly arranged, i.e. which are irregularly spaced, or randomly oriented, or both. In principle, the amount of crystalline material could be assessed by any one of a number of methods, both physical and chemical, depending on the differences between these two arrangements. Clearly the crystalline portion will be more resistant to chemical attack, have a higher density, less dye and water absorption, &c., and for each of these differences a method could be, and generally has been, devised. It is not proposed here to say anything as to the relative merits of these methods except that chemical methods, involving as they do the progressive removal of the non-crystalline fraction and therefore a continuous change in the nature of the experimental material, cannot be expected to yield results as reliable as those obtained by purely physical methods which leave the material at the end of the observations in precisely the same state as the original.

It should, however, be noted in addition that the division of cellulose into two fractions in this way is quite arbitrary; it seems highly probable that there exists a complete gradation of order from the chains within a lattice to the 'free' chains sufficiently far removed from these strictly ordered regions. The position at which the line is drawn to separate crystalline from non-crystalline depends therefore in no small degree on the method of investigation, and it should not be expected that different methods will yield the same figure any more than different methods of measuring molecular weights will give the same answer for cellulose. A forceful example of this is provided by the consideration that a parallel array of cellulose chains could appear highly crystalline by optical methods whether the chains were regularly spaced or not, provided they were not too far separated; the crystallinity determined by an X-ray method, on the other hand, depends entirely on the spatial arrangement of the chains and could therefore be zero. An example of the former case has already appeared (Preston and Middlebrook, 1949). The method used in this investigation limits the crystalline fraction to those chains only which take part in the formation of a regular crystalline lattice. The rest is then classed as non-crystalline.

In the present investigation three groups of samples were chosen as constituting cell types whose structure is rather well known and which are of interest botanically.

1. Cell walls of *Valonia ventricosa* L. The material consists of pieces of wall cut from vesicles preserved for some time in formalin-alcohol. After a preliminary washing in water these were boiled in 0.5 per cent. ammonium oxalate to remove any pectic substances, but no further purification was attempted in view of the present lack of knowledge concerning the chemical composition of the *Valonia* wall.

2. *Pinus radiata* wood.¹ Samples were taken from the 5th, 10th, and 15th annual rings (labelled V, X, and XV in Table I, section 2) and from each of

¹ These samples were prepared by Dr. A. B. Wardrop of the Forest Products Division, Melbourne, Australia, while working in my laboratory at Leeds.

these powders of Cross and Bevan cellulose were prepared. These were therefore free of lignin and pectin.

3. *Bamboo spp.*¹ Cross and Bevan cellulose of four species were prepared in the form of intact though separated fibres, and, with one species (*Dendrocalamus strictus*), the fibre mass was separated into three length classes by mechanical sieves.² The length classes are listed as I, II, and III, and A, B, C in Table I, section 2, and the corresponding lengths are as follows:

I. <i>Melocanna bambusoides</i> , 1.83 ± 0.09 mm.	A, 1.51 ± 0.08 mm.
II. <i>Bambusa acundinacea</i> , 2.86 ± 0.09 mm.	B, 2.35 ± 0.08 mm.
III. <i>Bambusa polymorpha</i> , 3.59 ± 0.13 mm.	C, 3.57 ± 0.11 mm.

A, B, and C refer to *D. strictus*.

Among the material perhaps *Valonia* may be considered first, since this alga has been a centre of interest for so long and since attention has once more been called to its importance in wall studies in a recent series of observations under the electron microscope (Preston *et al.*, 1948). Several features of the wall of this alga had led us to the conclusion that it must have a high crystalline-non-crystalline ratio. Among the earlier observations leading to this view may be cited the low swelling in sulphuric acid, the extreme difficulty in staining with iodine and sulphuric acid, lack of swelling in cuprammonium, and low permeability to solutes. None of these were, of course, convincing of themselves, but together they did give an impression of an unusually crystalline material. The observation that under the electron microscope the wall appears to be composed of fibrils some 250–300 Å diameter compares favourably, however, with a micelle size of some 200 Å which can be determined from the X-ray diagram. This latter figure is, of course, quite unreliable, but again gives the impression that the whole of the cellulose is confined to the fibrils and that these may be almost completely crystalline.

The other specimens which come under review here form a rather coherent group, and their interest and the reason for their study lies in this. It is now quite clear that some features of the organization of the cellulose in the secondary wall of elongated cells depend in no small measure on the dimensions of the cell. In all such cells the cellulose chains can be considered as forming a spiral³ round the cell, and it is found that the angle between the spiral winding and the length of the cell is a function of both the length and the breadth of a cell. Thus, to take the case of the tracheides of conifer wood, which were the first in which this relation was noted, it is now well established that the average length L of the tracheides in any one annual ring of a conifer stem is related to the spiral angle θ by a relation of the type (Preston, 1934),

$$L = K \cot \theta,$$

¹ These were prepared by Dr. K. Singh, Forest Research Institute, Dehra Dun, India, while working in my laboratory at Leeds.

² The separation of the fibres was done for me by Mr. D. A. Clark of Washington, U.S.A., to whom my thanks are due.

³ Strictly a helix, but the term *spiral* is so widespread in botanical studies that its use is retained here to avoid confusion.

where K is a constant, over a number of annual rings. The steadily increasing average length of the tracheides in passing from inner and outer rings at the same level in such a stem arises because the cells of the cambium which, by regular longitudinal tangential division, give rise to the tracheides, are themselves elongating, and they continue to do so for periods up to 60 years or more. It was therefore natural at first to conclude that the steepening of the spiral arose through the same agency. More recent investigation has shown, however, that this conception was in error. We now know that the primary wall which surrounds the cambial cell during its expansion contains cellulose chains lying almost transversely, and not in a steep spiral, and that this orientation is invariate with cell length (Preston, 1947; Preston and Wardrop, 1949 *b*). Again, it has appeared that if individual tracheides are studied, then irrespective of the position of origin of the tracheide in the stem, and therefore irrespective of the cambial cell from which it was derived, a similar relation holds good. Now, however, that more numerous figures can be obtained it is clear that the relation is of the type

$$L = A + B \cot \theta_s,$$

where A and B are constants and θ_s is the spiral angle of a tracheide corrected to standard breadth (Preston and Singh, 1950). If the breadth of the cell is also included in the formula, then the relation becomes of the type

$$L = A + B\sqrt{\{(b)^2 \operatorname{cosec}^2 \theta - 1\}},$$

where b is the breadth.

In the course of an investigation on bamboo fibres it has appeared that a similar relation holds with these fibres too, in spite of their very different method of development (Preston and Singh, 1950) and the same must apply also to sisal fibres (Preston and Middlebrook, 1949). Similarly, work in other laboratories has shown that cell length and spiral angle is related in some way, though the precise relation has not been specified; this applies both to cotton hairs (Meredith, 1946) and to some monocotyledonous fibres other than bamboo (Meeuse, 1938). In bamboo the density of the wall material is related to cell length (Preston and Singh, *unpub.*), and it seems probable, from the fact that the bulk density of wood varies regularly with distance from the stem centre at any level, that the same applies to wood. There is here, therefore, a strong suggestion that the crystallinity might be a function of length.

Now it must be remembered that the deposition of these secondary walls occurs after the bulk of the expansion of a cell is completed, so that there can be no direct connexion between elongation and wall structure such as would be analogous to the extension of a spiral spring. The connexion between wall structure and dimensions is therefore a puzzling one, whose explanation would go far towards yielding understanding of wall deposition generally. Any further information we can add to the nature of this connexion is therefore very welcome, and it is for this reason that the present investigation was attempted. The results are by no means so conclusive as could have been

hoped, but they are published at this time because they do indicate the need for further research and point out the lines along which future work should be prosecuted.

2. RESULTS OF X-RAY INVESTIGATION (by P. H. HERMANS and A. WEIDINGER)

The method used consists of intensity measurements on photographs taken with strictly monochromatized copper radiation from standardized pellets of fibres with random orientation (Hermans and Weidinger, 1949). Radial photometer curves were taken and reduced to equal intensity of primary radiation and irradiated mass.

A typical example of a standardized photometer curve is given in Fig. 1. The crystalline interferences, such as A_1 and A_4 , are separated from the diffuse background by the broken curve beginning at the point of inflexion a and asymptotic to the background at the outer edge of the diagram. The total integrated intensity I_{cr} of the crystalline peaks (surface above the broken line), expressed in arbitrary units, is considered as a measure of the crystalline portion. The height Am of the flat maximum of the diffuse background, after correction for air scattering (dotted line) and incoherent radiation (lower hatched area), also expressed in arbitrary units, is considered as a measure of the non-crystalline portion. The values of I_{cr} and Am found for the fibres investigated in this research are listed in Table I together with the percentages of crystallinity derived therefrom by two methods. (For the samples marked with an asterisk the average of two well-conforming determinations is given.)

TABLE I

Specimen	I_{cr}	Am	Crystalline percentage		
			From Fig. 1	From I_{cr}	
<i>Valonia</i> *	194	30	65-70	70	
					Sorption ratio at 65 per cent. R.H.
Cross and Bevan V*	206	31	70	73	1.38
" " " X	171	41	55-60	61	1.67
" " " XV. . . .	160	39	55-60	57	1.55
Bamboo I*	140	42	50	50	
" II	155	39	55	55	
" III	177	39	60	64	
					Density
<i>D. strictus</i> A*	179	40	60	64	1.528
" B	179	40	60	64	1.531
" C	163	39	55-60	58	1.535
					Sorption ratio
Bacterial cellulose	119	58	40	—	1.22
Ramie and cotton	198	28	69	—	1.0
Viscose rayon	113	56	39	—	1.85-1.95

* Listed in order of increasing cell length—see Section I.

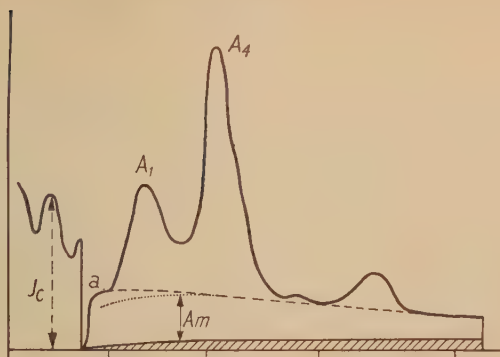


FIG. 1. Standardized photometer curve of ramie fibres randomly orientated. (J_c is the interference of an inorganic comparison substance located near the centre of the diagram and serving to measure the intensity of the primary beam of X-rays. It serves to standardize all photographs taken to equal amount of primary radiation.)

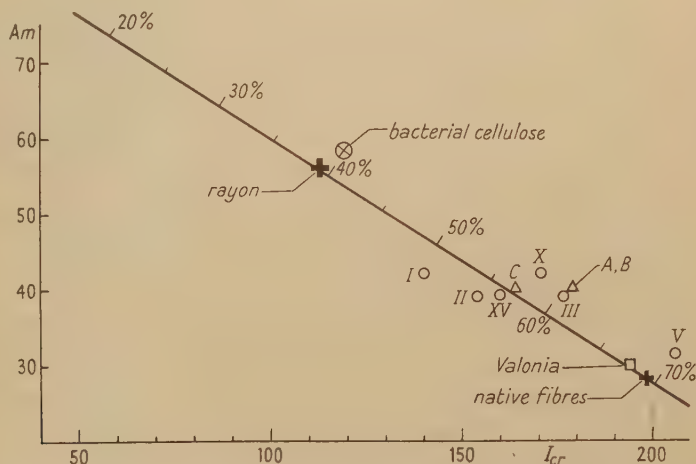


FIG. 2. Crystallinity nomograph plotted from the I_{cr} and Am values from Table I. Percentage of crystalline portion indicated along oblique line. $\circ$ (V, X, XV) *Pinus radiata* Cross and Bevan celluloses, $\circ$ (I, II, III) Bamboo, Δ *Dendrocalamus strictus*.

In the first method the experimental values of I_{cr} and Am are plotted against each other (see Fig. 2). It is easy to see that the points should then fall on a straight line. If the crystalline fraction of a sample is p and its non-crystalline fraction $(1-p)$, then the assumed proportionality of these quantities with I_{cr} and Am can be thus expressed:

$$I_{cr} = Bp \text{ and } Am = A(1-p).$$

Substituting x and y for I_{cr} and Am respectively we get:

$$x = Bp \text{ and } y = A(1-p).$$

From this follows

$$Ax + By = AB,$$

which is the equation of a straight line intersecting the x and y axes at $x = B$ and $y = A$. The values of A and B can thus be evaluated from observations on samples having widely different values of p , and to every point of the line can be assigned a known value of p . This is indicated as a crystallinity scale along the line in Fig. 2. The crosses represent the average values found for viscose rayon (40 per cent.) and for purified cotton, ramie, and flax fibres (70 per cent.), which can all be considered as samples of pure cellulose.

The points found for a number of other cellulosic objects of technical interest (not recorded in Fig. 1) also fall on or very near this line (Hermans and Weidinger, 1948, 1949).

It is seen that some of the fibres listed in Table I yield some points lying somewhat off the line. From the location of these points only the approximate crystallinity figures as given in the third column of the table can be derived.

The reason for the deviation from the straight line may be the presence of unknown amounts of impurities in the fibres which affect the value of Am . This is suggested by a recent investigation on jute fibres containing a large proportion of alien substances (Sen and Hermans). It also followed from that work that in such cases the computation of the crystallinity figure from I_{cr} alone, using the relevant well-established figures found for rayon, cotton, and ramie as a guide, gives the more reliable values. The values so computed are given in the fourth column of Table I. The experimental error in these figures is estimated to be about plus or minus 3 per cent.

In the last column of the table the sorption of water vapour at 65 per cent. relative humidity (expressed in that of cotton as a unit) is given for the Cross and Bevan celluloses. For the *Dendrocalamus* samples the density values (determined in R. D. Preston's laboratory) are listed.

For *Valonia* the sorption was also determined and appeared to be entirely anomalous, in that the sorption ratios increased considerably towards lower relative humidities, whereas the majority of cellulose samples yield almost constant figures, independent of the relative humidity. In Table II the relevant data are collected together with those previously found for bacterial cellulose which show the same anomaly.

TABLE II

Water absorption of Valonia cell wall and of bacterial cellulose at various relative humidities and their sorption ratio as compared to standard cotton cellulose

% relative humidity	Cotton % H ₂ O	<i>Valonia</i> % H ₂ O	ratio	Bacterial Cellulose	
				% H ₂ O	Ratio
10	2.10	3.07	1.46	4.20	2.0
17.5	2.80	3.46	1.24	5.10	1.79
35	4.30	4.10	0.95	5.93	1.38
65	7.00	5.87	0.84	8.55	1.22

DISCUSSION (by R. D. PRESTON)

It is at first sight somewhat disappointing to find the crystallinity of the *Valonia* cell wall appearing somewhat lower than that of the native fibres, but an explanation of this could readily be offered in terms of the probable impurity of the cellulose in these samples. The anomalies in the water sorption (Table II) parallel rather closely the anomalies found in bacterial cellulose in spite of the very different proportion of crystalline material estimated to be present. It seems, therefore, very likely that some non-cellulosic substance present is interfering both with the determinations of crystalline-non-crystalline ratios by the X-ray method and with the sorption of water; although it remains just possible that some particular structural principle is involved.

The other samples behave fairly normally in that the figures for percentage crystallinity are paralleled by those for water sorption and there seems little reason to suspect any major interference by foreign substances.

The wood samples in particular are interesting and involve some most intriguing possibilities, perhaps particularly since the gradation in the crystalline ratio is in the opposite direction to that expected! The clear separation between the wood of annual ring V and the rings X and XV makes it quite certain that in this particular material the former sample has much the higher crystalline-non-crystalline ratio; and, though rings X and XV are too close together in the series to warrant any definite statement, nevertheless the indication is that ring X is rather more crystalline than ring XV. Remembering that in the order V, X, XV the wood consists of tracheides of increasing length wound with molecular spirals of cellulose of increasing steepness, it is then clear that the flatter spirals contain the higher percentage of crystalline cellulose. Further comment is clearly undesirable until more material is investigated, but certainly there is every encouragement here for further work, all the more particularly in view of the results of the bamboo investigation.

The two sets of bamboo samples gave, unfortunately, somewhat discordant results, and the evidence so far as it goes is that this, too, may be attributed to the presence of unknown and variable amounts of impurities. This applies more particularly to the samples A, B, and C, where the crystalline ratios are not significantly different in spite of the different densities of the samples. In the series I, II, and III, on the other hand, there is clear evidence of a gradation in crystallinity which this time parallels the increase in length. This is perhaps the most interesting result of the whole investigation, since in the wood samples the correlation is in the opposite sense.

It is therefore clear, even from this very preliminary survey, that there is scope for a very thorough-going investigation of the crystallinity in botanical material and, if it has done nothing else, this work has shown that in future investigations strict attention must be paid to purity in the samples presented to the X-ray spectrometer.

LITERATURE CITED

- FREY-WYSSLING, A. (1948). *Submicroscopic morphology of protoplasm and its derivatives*. Elsevier, New York, Amsterdam.
- HERMANS, P. H. and WEIDINGER, A. (1948). *J. Applied Phys.* **19**, 491.
- (1948). *Bull. Soc. Chim. Belge*, **57**, 123.
- (1949). *J. Polymer Sci.* **4**, 135.
- MEEUSE, A. D. J. (1938). *Rec. Trav. Botan. Neerland.* **35**, 288.
- MEREDITH, A. (1946). *J. Text. Inst.* **37**, T 205.
- NORMAN, A. G. (1935). *Biochemistry of cellulose*, &c. London.
- PRESTON, R. D. (1934). *Phil. Trans. Roy. Soc. Lond. B* **224**, 131.
- (1947). *Proc. Roy. Soc. Lond. B* **134**, 202.
- (1949). *Biochim. et Biophys. Acta*, **2**, 370.
- and MIDDLEBROOK, M. (1949). *J. Text. Inst.* (In the press.)
- , NICOLAI, E., REED, R., and MILLARD, A. (1948). *Nature*, **162**, 665.
- and SINGH, K. (1950). *J. Exp. Bot.* **1**, 214.
- and WARDROP, A. B. (1949 a). *Biochim. et Biophys. Acta*, **3**, 549.
- (1949 b). *Ibid.* **3**, 549.
- SEN, M. K., and HERMANS, P. H. *Rec. trav. chim.* (In preparation.)

Studies in the Physiology of the Onion Plant

IV. THE INFLUENCE OF DAY-LENGTH AND TEMPERATURE ON THE FLOWERING OF THE ONION PLANT

M. HOLDSWORTH AND O. V. S. HEATH

*Research Institute of Plant Physiology, Imperial College of Science and Technology,
London, S.W. 7*

INTRODUCTION

GARNER and Allard reported in 1923 that the flowering of onion plants was affected by day-length: inflorescences emerged both in long summer days and in day-lengths artificially shortened to 5 hours but not in intermediate day-lengths. The plants were, however, grown from sets and it is now evident that day length can only have affected inflorescence emergence, for initiation of inflorescence primordia would have occurred in the sets during storage, that is before the experiments were begun (Thompson and Smith, 1938; Heath and Mathur, 1944). The original production of sets must have taken place in long days, for bulbing does not occur at all in days shorter than a certain minimum (Garner and Allard, 1923; McClelland, 1928; Magruder and Allard, 1937; Thompson and Smith, 1938; Heath, 1943*b*). It is necessary, therefore, to consider whether the initiation of inflorescences is, in fact, independent of day-length—since the initiation in stored sets usually occurs in the dark—or whether initiation is an after-effect of the long days prevailing during the production of sets. To decide these questions onion plants must be raised from seed and given appropriate day-length treatments from the outset.

The effect of temperature on the initiation of inflorescence primordia during storage of onion sets is a question of some economic importance and has been investigated by a number of workers. The heat treatment of onion sets to prevent 'bolting' in the plants subsequently grown from them is said to be a traditional part of onion-set culture in Hungary. Thompson and Smith (1938) found that storage at temperatures of 60–70° F. did much to prevent bolting and Heath and Mathur (1944) and Hartsema (1947) have shown that this is an effect on inflorescence *initiation*, which tends to be suppressed both by high temperatures and by temperatures about 0° C. Heath (1943*b*) also obtained some evidence that high-temperature conditions during the production of sets suppresses inflorescence initiation independently of the storage temperature.

The effects of day-length and temperature on inflorescence emergence are made complex and, under most conditions, disguised, by the effects of these factors on bulbing, for the onset of bulbing appears in itself to inhibit inflorescence emergence (Heath and Mathur, 1944). Bulb formation is encouraged both by long days and high temperatures (Thompson and Smith,

1938; Heath, 1943*b*), and when both these conditions are operating inflorescence emergence is prevented. It seems clear, however, that high temperature (20° C. or above) alone is sufficient to prevent inflorescence emergence, for such temperatures control bolting when applied under short-day conditions, in which bulbing does not occur (Heath, 1943*b*; Heath and Mathur, 1944).

EXPERIMENTAL

TABLE I

Synopsis of Experiments

Symbols: For day-length, D = continuous darkness, L = long (16½ hour), S = short (11 or 11½ hour), N = natural; for temperature, *l* = low and *h* = high temperature section of greenhouse, *o* = outdoor, *st* = storage.

Experiment	Variety	Treatment Nos.	Day-length treatment				Temperature treatment			
			1st sum- mer	1st win- ter	2nd sum- mer	2nd win- ter	1st sum- mer	1st win- ter	2nd sum- mer	2nd win- ter
I (1941-3)	Bedford- shire	1 & 3	N	D	S	S	<i>o</i>	<i>st</i>	<i>l</i> & <i>h</i>	
	Champion (sets)	2 & 4	N	D	S	L	<i>o</i>	<i>st</i>	throughout	
II (1943-4)	Ailsa	1	—	S	S	S	—	} <i>l</i> throughout		
	Craig (seed)	2	—	S	L	S	—			
III (1942-4)	Ebenezer (seed)	1	—	L	L	L	—	<i>l</i> throughout		
IV (1943-5)	Stock 28 (seed)	1	S	S	S	S	} <i>l</i> throughout			
		2	L	L	L	L				
		3	S	S	S	L				
		4	S	S	L	S				
		5	S	S	L	L				
		6	—	—	S	S	—	—	<i>l</i> throughout	
		7	—	—	S	S	—	—	} <i>h</i> throughout	
		8	—	—	L	L	—	—		
V (1943-5)	Brown	1	N	D	S	S	<i>o</i>	<i>st</i>	} <i>l</i> throughout	
	Globe (sets)	2	N	D	S	L	<i>o</i>	<i>st</i>		
VI (1944-6)	Brown	1 & 4	—	S	S	S	—	} <i>l</i> & <i>h</i> throughout		
	Globe (sets)	2 & 5	—	L	L	L	—			
		3	—	S	S	L	—	<i>l</i> throughout		

Note. The periods, and therefore the mean temperatures, covered by 'summer' and 'winter' respectively differed considerably between the various experiments and reference must be made to the accounts of these for details.

In Expt. I, described below, long-day conditions were given at temperatures so low that bulbing was considerably delayed, in order that the *long-day* effect on bolting might be separated from the bulbing effects. This experiment has already been briefly described (Heath and Holdsworth, 1943), and as the conclusions have been confirmed by the results of some experiments of Scully *et al.* (1945) it is convenient to summarize them before describing the experimental details: Inflorescence initiation can occur in plants which are not in the bulbed condition. The temperature, however, must be similar (*c.* 13° C.)

to that at which initiation normally occurs in storage. The plants used in Expt. I had originally been produced from sets and, therefore, on the basis of this experiment alone the possibility remains that long days are necessary at some period in the onion plant's development for flowering to occur. In the absence of bulbing, inflorescence emergence is more rapid in long than in short days.

In Table I 'long days' means $16\frac{1}{2}$ hours' light daily and 'short days' 11 hours, except in Expt. I and the early part of Expt. II in which the 'short-day' plants received $11\frac{1}{2}$ hours' light daily. The day-lengths were controlled as described elsewhere (Heath, 1943*b*). The plants were grown in tins of soil, $5\frac{3}{4} \times 5\frac{3}{4} \times 8$ in. deep, with five plants to each except where gaps had occurred.

All experiments except II and III tested the effect of day-length on emergence of inflorescences in the absence of bulbing, by comparing long and short days in the final winter. Expts. I, IV, and VI showed the effect of temperature on inflorescence initiation. Expts. II and IV tested the effect on initiation and subsequent inflorescence emergence of allowing the plants to bulb in long days in the summer preceding the final winter. Expts. II, IV, V, and VI showed whether long days were essential for inflorescence initiation or emergence; Expts. III, IV, and VI did the same for short days.

Experiment I. 1941-3. Variety 'Bedfordshire Champion'

For the purpose of a large factorial experiment on the effects of storage temperature on the subsequent flowering of onion plants grown from sets, plants of variety 'Bedfordshire Champion' were grown from 17.3.42 in the greenhouse in short days at two temperatures (means 18° and 26° C.). Only two plants flowered in the whole experiment, and dissections of comparable sets pickled at the time of planting (17.3.42) showed that none had initiated inflorescences during storage irrespective of temperature. The sets were very small (none greater than 2 gm. in weight) and this was no doubt the reason for the failure to flower, as bolting is dependent on set size (Thompson and Smith, 1938; Holdsworth, 1945*a*; Heath *et al* 1947). On 6.10.42 the plants were divided at random into two lots and one lot was transferred to long days. The two greenhouse temperature treatments were continued as before, but with the falling outdoor temperatures, as the winter advanced, the mean temperatures of the respective treatments were lower than during the summer (means 14° and 24° C.; Fig. 1*a*). From 15.12.42 onwards inflorescences appeared in the long-day, low-temperature treatment. By 26.1.43 about one-fifth of the plants had bolted in this treatment, and there being no indication of bolting in any of the other treatments all the plants were pickled and later dissected. Throughout the experiment records of leaf number, bulbing, and bolting had been made fortnightly.

Results. As recorded above, inflorescences emerged only in the long-day, low-temperature treatment. Ten weeks after transference to long days 7 per cent. of the plants in this treatment had bolted and in subsequent fortnights the percentage increased to 12, 19, and 21 respectively. The dissections for the

last of these counting dates (26.1.43) showed, however, that although no inflorescences had emerged in the short-day, low-temperature treatment the total percentage of plants containing inflorescences or inflorescence initials was the same as in the long-day, low-temperature treatment, viz. 65 per cent.

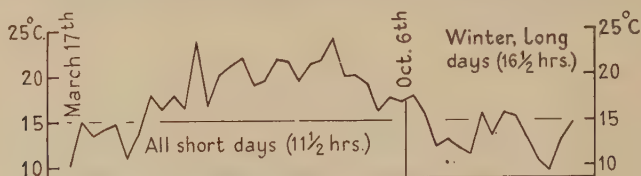


FIG. 1a.

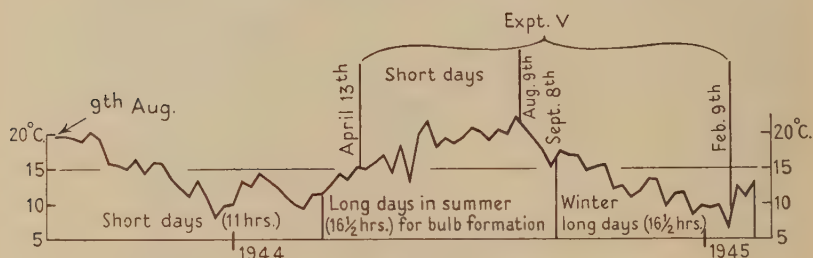


FIG. 1b.



FIG. 1c.

FIG. 1. Weekly mean air temperatures in low-temperature part of greenhouse. a. Expt. I (17.3.42–26.1.43); b. Expts. IV (9.8.43–23.2.45) and V (13.4.44–9.2.45); c. Expt. VI (13.9.44–9.4.46).

(Table II). The distribution of scape development of the inflorescences among plants in the two treatments was, however, very different (Fig. 2). The plants at high temperature in either day-length were all vegetative.

The effects of the treatments on bulbing are also shown in Table II. Bulbing began earliest in the high-temperature, long-day treatment and here all the plants had bulbed and 67 per cent. ripened by the end of the experiment. In the corresponding low-temperature treatment the onset of bulbing was greatly delayed. In either short-day treatment the amount of bulbing was negligible.

From the progress of leaf emergence shown in Table II it is evident that in the high-temperature treatment the rate of emergence began to fall soon after transfer to long days (6.10.42). This fall and cessation at high temperature of leaf emergence in long days is undoubtedly an indirect effect of bulb-

TABLE II

Experiment I. 1941-3. 'Bedfordshire Champion'

Treatment				Data for 26.1.43				Average no. of leaves emerged since planting of sets (17.3.42)		
No.	Temp.	Day-length		Total no. of plants	% with infis.	% with emerged infis.	% bulbed			
		Hrs.	Period of application							
1	Low	11½	17.3.42-26.1.43	60	65	0	0	19.7	23.3	26.3
2	Low	11½	17.3.42-6.10.42	57	65	21	40	19.7	23.9	26.1
		16½	6.10.42-26.1.43							
3	High	11½	17.3.42-26.1.43	65	0	0	3	20.9	24.5	28.2
4	High	11½	17.3.42-6.10.42	58	0	0	100	20.8	23.5	23.5
		16½	6.10.42-26.1.43							

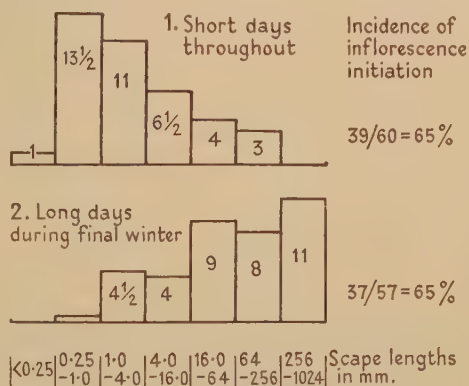


Fig. 2. Expt. I. 1942-3. Distribution of scape lengths at end of experiment (26.1.43). (Low temperature treatments only; high-temperature plants were all vegetative.)

ing, for leaf emergence is known to cease shortly after bulbing onset (Heath, 1943*b*). With the production of inflorescence primordia, also, leaf emergence ceases temporarily until growth is resumed by an axillary bud. The average total number of leaves produced since the sets were planted and before inflorescence initiation was 30.0 in short days and 29.0 in long days.

Experiment II. 1943-4. Variety 'Ailsa Craig Prizetaker'

As Expt. I was not originally set up to test the effect of day-length treatments at winter temperatures, some important data could not be obtained from it. Especially it seemed that the total number of leaves produced before inflorescence initiation would probably be of the greatest interest in view of the relationship between leaf number and the production of ears in cereals (Purvis and Gregory 1937). Therefore some experiments in this series were begun from seed, and leaf production followed throughout. Expt. II of 1943 was the first of these.

It was intended to grow as usual five plants in each tin, and as the experi-

ment could be expected to last more than a year a considerable number of extra plants had to be allowed during the early stages. In each position in the tins, therefore, ten seeds were sown and the seedlings progressively thinned out until only one plant remained in each position. In spite of this precaution a considerable further number of plants was lost during the final winter of the experiment; it has been found throughout the experiments that follow that plants are very susceptible to *Botrytis* attack during the winter, perhaps because the low light intensity causes etiolation.

TABLE III
Experiment II. 1942-3, 'Ailsa Craig' Prizetaker'

Treatment		17.4.44					Emerged leaves, at time of bulbing and just before first bolting	
		Total no. of plants	% with infls.	% with emerged infls.	Emerged leaves preceding infl.	Emerged leaves + scales preceding infl.	31.5.43	7.2.44
1	S 29.1.43-17.4.44	25	100	100	32.5	32.5	8.9	31.6
	S 29.1.43-10.3.43							
2	L 10.3.43-31.7.43	20	90	30	24.6	29.6	8.5	21.7
	S 31.7.43-17.4.44							
	S 17.4.44							

Values linked by a bracket [differ significantly at $P = 0.1$

The seed was sown on 29.1.43, and until 10.3.43 all the seedlings received the same treatment: low temperature and short days. The latter were the natural winter day-lengths received on the open benches of the greenhouse. On 10.3.43 the tins of seedlings were divided into 4 lots, at random, of 4 tins each (20 plants). Two lots went to duplicate day-length trolleys receiving $11\frac{1}{2}$ -hour days, and the other two to $16\frac{1}{2}$ -hour days. The latter treatment was continued until all the plants had bulbed (31.7.43) and then these tins were also put into $11\frac{1}{2}$ -hour days alongside the corresponding tins kept in short days throughout. A few of the bulbs produced were sprouting by this time and new growth became general shortly after the transfer (by 12.9.43). The short day-length was changed to 11 hours instead of $11\frac{1}{2}$ on 16 August, and this has been used for all subsequent experiments. The experiment was continued until 10.3.44 when losses by fungal attack were becoming severe. The plants severely attacked were then pickled for dissection and those remaining were pickled on 17 April. The average temperature during the first winter (29.1.43 to 10.3.43) was 12.5°C. ; during the summer (until 21.9.43) 18.9°C. and during the final winter (22.9.43 to 19.3.44), 12.6°C. The trend during this last period can be seen in the early part of Fig. 1b.

Results. The earliest inflorescences to emerge appeared in the short-day-throughout treatment; one was produced by 22.11.43, but this was an isolated case until 18.2.44, after which bolting became general. The first inflorescence

among the plants caused to bulb in the preceding summer emerged on 3.4.44, and only 30 per cent. of these plants had bolted by the end of the experiment (Table III). It was therefore evident without mathematical analysis that previous bulbing had delayed emergence. On the other hand, bulbing caused inflorescence initiation after a smaller total number of leaves had emerged (Table III).

A subsidiary effect was shown by the plants which bulbed in the summer

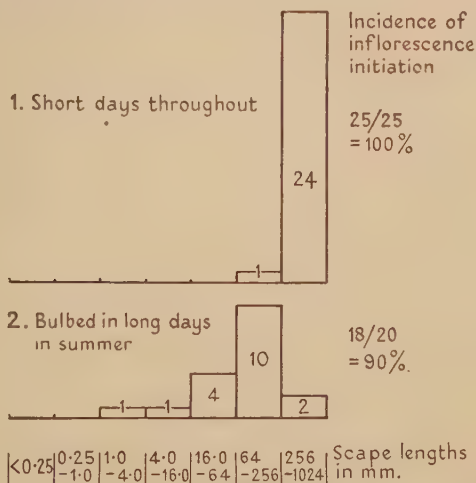


FIG. 3. Expt. II. 1943-4. Distribution of scape lengths at end of experiment (17.4.44).

in that 70.3 per cent. of them developed axillary branches after sprouting, whereas the main axis continued alone throughout the experiment in the short-day plants.

The distribution of scape lengths, including both emerged inflorescences and unemerged initials, is shown in Fig. 3.

Experiment III. 1942-4. Variety 'Ebenezer'

On 25.11.42 seed was sown in 16½-hour days at low temperature for use in an experiment on bulbing. Twenty of the small bulbs produced were, after ripening, replanted in four tins on 19.7.43, and the day-length was kept constant at 16½ hours until the end of the experiment (17.1.44); the remaining plants were then pickled for dissection. The average temperature during the first winter of this experiment was 13.2° C., during the summer 19.1° C., and during the final winter 13.0° C. (25.11.42–21.3.43, 22.3.43–21.9.43, 22.9.43–17.1.44 respectively).

Results. The average date of germination was 5.12.42. In the first production of bulbs from seed 24 seedlings were grown in each tin and although from 6 to 11 (average 7.9) leaves had been produced before ripening, the

bulbs were small because of crowding. The mean date of the first bulbing was 3.5.43. By 20.9.43 about half of the plants had bulbed a second time after producing another 6–9 leaves. The average temperature in the greenhouse was now falling and the rest of the plants did not bulb until very near the end of the experiment, by which time 65 per cent. of the plants were bulbed and two were sprouting afresh. The first plant to produce an inflorescence did so on 1.1.44, and one other plant bolted before the experiment was terminated. On dissection only two more plants were found to have initiated inflorescences (21 per cent.) and the average number of leaves and scales produced before the inflorescence was 28.8 (S.E. ± 0.65 ; range 27–30). The average total number of leaves formed by non-flowering plants before the end of the experiment was 32.9 ± 0.52 with a range of 29–37. In arriving at these figures it has been assumed that five scale leaves (not counted) were formed at the first bulbing (Heath, 1945).

Experiment IV. 1943–5. Variety 'Cambridge Stock 28'

Plants were grown from seed and the whole experiment originally consisted of 32 tins, which were sown with 8 seeds in each of the five positions on 9.8.43. The number of seedlings was reduced to 3 in each position on 3.12.43, to 2 on 11.2.44, and to 1 on 10.3.44. From then on leaf-counts were made fortnightly. Originally there were 20 tins for treatments 1, 3, 4, and 5

TABLE IV
Experiment IV. 1943–5. 'Stock 28'

Treatment			23.2.45					
No.	Temp.	Day length and period of application	Total no. of plants	% with infls.	% with infls. emerged	Emerged leaves preceding infl.	Emerged leaves + scales preceding infl.	Bulb/neck ratio
1	Low	S 9.8.43–23.2.45	22	82	0	33.0	34.3	1.12*
2	Low	L 9.8.43–23.2.45	19	84	11	24.9	35.2	1.44
3	Low	S 9.8.43–8.9.44	22	77	61	33.2	34.1	1.40
		L 8.9.44–23.2.45						
4	Low	S 9.8.43–10.3.44	22	77	0	26.2	31.8	1.14*
		L 10.3.44–8.9.44						
		S 8.9.44–23.2.45						
5	Low	S 9.8.43–10.3.44	22	82	11	24.2	29.3	1.50
		L 10.3.44–23.2.45						
6	Low	S 2.3.44–23.2.45	20	58	0	22.0	24.2	1.14*
7	High	S 3.3.44–23.2.45	4	0	0	—	—	—
8	High	L 3.3.44–23.2.45	14	0	0	—	—	2.03

* No bulbing.

(Table IV) and 4 tins each for treatment 2 and also short-day, high-temperature and long-day, high-temperature treatments. Unfortunately, so many seedlings were lost in the last two treatments during the first winter that this part of the experiment was repeated by the treatments 6, 7, and 8 (Table IV)

which were resown on 3.4.44. The low-temperature, short-day plants were all exposed to natural short days in the greenhouse from 13.1.44 until 10.3.44 because of lack of space on the day-length trolleys. On that date ten of these tins were transferred to long-day, low-temperature and there allowed to bulb and ripen (treatments 4 and 5). Half of these were returned to short days on 8.9.44 (treatment 4), and at the same time a further five short-day, low-temperature tins were transferred to long days (treatment 3). Thus the experiment comprises, at the low temperature, plants which after an autumn and winter in short days were grown at all combinations of long and short days in the summer and long and short days in the winter; also plants grown in long days throughout (treatment 2). The high-temperature treatments (long and short days throughout only) were sown seven months later, in early spring instead of late summer, and were therefore not comparable with the low-temperature treatments (1-5). One of the latter was repeated in treatment 6 to compare with the high-temperature treatments and also to give information as to the effects of age and size of plant by comparison with treatment 1.

Results. The average date of germination in treatments 1-5 was 16.8.43 and in treatment 6, 17.3.44. A single inflorescence appeared on 31.7.44 in treatment 1, but no further inflorescences appeared until 22.12.44, the first being in treatment 3. Further inflorescences appeared frequently until 23.2.45, when the plants were pickled for subsequent dissection. The percentages of plants with emerged inflorescences at the end of the experiment were as shown in Table IV, where it is seen that no inflorescences had appeared in either high-temperature treatment, nor in any plant which was in short days at the end of the experiment. Most bolting occurred, on the other hand, in treatment 3 which received long days in the final winter only.

It is seen that treatments 1-5 did not, however, differ greatly in the percentage of plants which had initiated inflorescences before the end of the experiment. The relative development of the inflorescences is indicated in Fig. 4.

In treatment 6, although no bolting had occurred by the end of the experiment, 58 per cent. of the plants had initiated inflorescences and one of these was approaching emergence. Although the progress towards flowering was appreciably less than in treatment 1, this effect was much smaller than might be expected to result from seven months' difference in age. In treatment 6, inflorescences were initiated after a much smaller number of leaves than in treatment 1 (Table IV).

The number of emerged leaves produced before the inflorescence was greatly reduced in those treatments (2, 4, and 5) where bulbing occurred during the summer. In short days at low temperature (treatments 1 and 6) no plants bulbed during the whole period of the experiment. In the plants exposed to long days in the final winter only (3), bulbing had begun in 35 per cent. at the end of the experiment. Where plants had bulbed in the summer it was often difficult to judge whether bulbing had begun again and the

measure of bulbing presented in the last column of Table IV is also more satisfactory for the other treatments as the measurements of diameter were made after removal of the old bulb scales at the time of dissection.

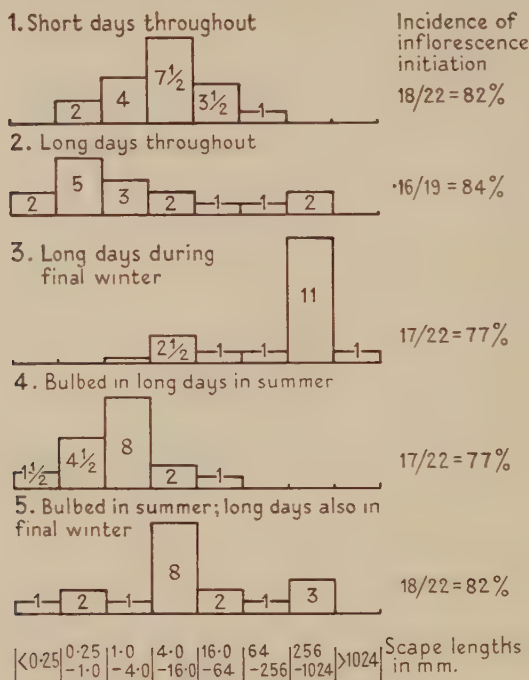


FIG. 4. Expt. IV. 1943-5. Distribution of scape lengths at end of experiment (23.2.45). (Low-temperature treatments only; high-temperature plants were all vegetative.)

Experiment V. 1943-5. Variety 'Brown Globe'

This experiment was set up to confirm the original experiment (Expt. I) performed with sets of var. 'Bedfordshire Champion', but without the complication of the preliminary differential storage treatments. The variety used was known to be a 'bolting' strain, but very small sets were chosen after ordinary storage temperature treatment. Comparable sets dissected at the end of storage (April 1944) were all in the vegetative condition. Twelve tins were planted with sets of the size range 0.7-1.0 g. on 13.4.44, and all the plants were grown through the summer in short days in the low-temperature part of the greenhouse. On 9.8.44 half of the tins, chosen at random, were transferred to long days and the experiment so continued until 9.2.45, when all plants remaining were harvested and pickled for dissection.

Results. No emerged inflorescences had been produced in the short-day treatment by the end of the experiment. In the long-day treatment 19 out of

30 or 63 per cent. of the plants had bolted by this time. There were no bulbed plants in the short-day treatment, but in long days a few plants had bulbed by 3.11.44, and by the end of the experiment 41 per cent. of the plants had new bulbs.

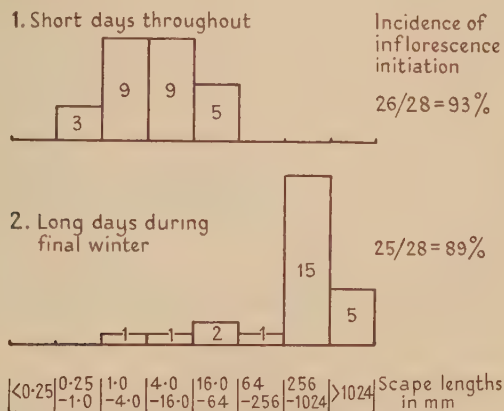


FIG. 5. Expt. V. 1944-5. Distribution of scape lengths at end of experiment (9.2.45).

On dissection it was found that the total number of plants in long days which had produced inflorescence initials was 25 (89 per cent.) and in short days 26 (93 per cent.). The distributions of scape lengths are shown in Fig. 5. The mean number of leaves preceding the inflorescence was 27.9 (24-31) in short days and 26.5 (24-30) in long days. The mean numbers of leaves produced by the plants which were still vegetative were 28.0 and 32.0 respectively.

Experiment VI. 1944-6. Variety 'Brown Globe'

In this experiment the plants were raised from seed and the design was similar to Expt. IV, but involved a smaller number of treatments (Table V). Seed was sown on 13.9.44, allowing 10 seeds to each of the 5 positions per tin. All the tins were placed in the high-temperature part of the greenhouse in natural day-lengths to germinate and on 29.9.44 were placed in their respective temperatures on the day-length trolleys, with the exception of the low-temperature, short-day treatments (3 and 5) which, for lack of space, had to remain in natural day-lengths for the winter. On 26.2.45 these also were accommodated on the day-length trolleys. As it had been found in previous experiments that the normal losses of plants during the winter were especially severe in the high-temperature treatments, the high-temperature treated seedlings were illuminated in dull weather by 1,000-watt lamps from 19.10.44. A contemporary test of the effect of such illumination at the same temperature (but in a different variety) showed a marked improvement in survival and a small but consistent hastening in emergence of the first three leaves. For the same reasons thinning of seedlings in the high-temperature treatments was

postponed compared with the low-temperature treatments which were thinned to a single plant per position on 1.5.45. On this date the high-temperature treatments were reduced to a maximum of 3 per position and later (12 June) the number was reduced to 5 per tin, leaving not more than 2 in each position. Considerable trouble was experienced with red spider mite in this experiment, especially in the high-temperature treatment. Several different measures were used in the attempt to control the pest and fumigation with methyl bromide proved partially successful. The use of a proprietary preparation of dicyclohexanol on 13.4.45 caused considerable leaf damage especially to the high-temperature plants, but all plants recovered without signs of permanent injury. Half the low-temperature, short-day tins were transferred to long days on 3.10.44 (treatment 3) and the experiment was continued until 12.2.46, when half of the plants (taken at random) from each of the low-temperature treatments were pickled for dissection. The remaining plants and all those of the high-temperature treatments were pickled on 9.4.46.

TABLE V
Experiment VI. 1944-6. 'Brown Globe'

Treatment			Data for 1st and 2nd samplings (12.2.46 and 9.4.46)				
No.	Temp.	Day length and period of application	Total no. of plants	% with infs.	% with infs. emerged	% bulbed	Bulb/ neck ratio
1	Low	S { 29.9.44-12.2.46	28	68	0	0	1.30
		29.9.44-9.4.46	25	88	16	0	1.26
2	Low	L { 29.9.44-12.2.46	16	31	0	56	3.32
		29.9.44-9.4.46	14	71	11	72	—
3	Low	S { 29.9.44-3.10.45	—	—	—	—	—
		L { 3.10.45-12.2.46	27	59	0	46	1.72
		3.10.45-9.4.46	21	24	24	91	2.62
4	High	S { 29.9.44-12.2.46	—	—	—	12	—
		29.9.44-9.4.46	33	0	0	15	—
5	High	L { 29.9.44-12.2.46	—	—	—	54	—
		29.9.44-9.4.46	35	0	0	79	—

Results. A small amount of bulbing occurred sporadically among the plants then in short days from 3.4.45. In the following weeks most of the bulbs produced were lost again and there is a possibility that the day-length treatments may at some time have been confused. In the subsequent thinning of the seedlings any plants which had shown pronounced bulbing were discarded. Otherwise no bulbing took place in the short-day treatments at low temperatures up to the end of the experiment, and in the high-temperature, short-day treatment only 5 of the plants were recorded as bulbed. With the plants kept in long days throughout, bulbing was first recorded in a few plants at both temperatures by 3.4.45; 50 per cent. of the plants were bulbed in high temperature by 17.4.45 and in low temperature by 1.5.45. Most of the plants had bulbed a second time at the low temperature by the end of the experiment (Table V) and in the high temperature some plants had bulbed again

twice before pickling. The bulbs in high temperature remained dormant longer on the average than did those in low temperature, and none were lost by rotting during dormancy.

The first inflorescence emerged on 5.2.46 among the plants transferred to long days for the winter.¹ By the time of the second pickling there were emerged inflorescences in all the low-temperature treatments (Table V), the

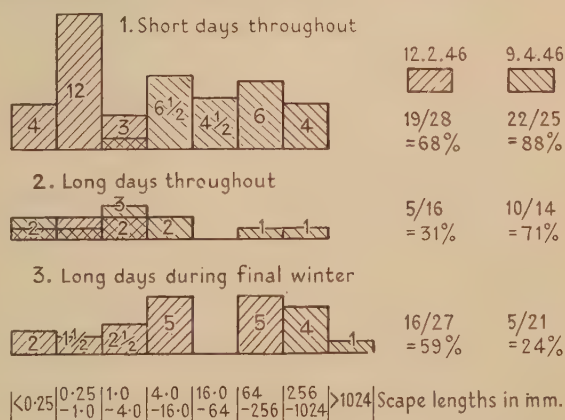


FIG. 6. Expt. VI. 1944-6. Distribution of scape lengths at 1st sampling (12.2.46) and at end of experiment (9.4.46). (Low-temperature treatments only; high-temperature plants were all vegetative.)

first in short days having appeared on 2.4.46. The number which emerged was, however, small in all treatments. No inflorescences at all appeared in the high-temperature part of the experiment, and on dissection inflorescences were found to be wholly absent from the plants grown at high temperatures. Of the plants from the low-temperature treatments, at the early sampling the percentage of plants with initiated inflorescences was similar in short days and in long days for the final winter treatments 1 and 3 (Table V). In the continuous long-day treatment, however, there were fewer inflorescences. By the time of the second sampling the percentage with inflorescences among the plants transferred to long days had apparently declined; indeed no inflorescences in addition to those already emerged were found in this sample on dissection. This is a very curious result and a possible reason for it is considered on p. 370. The scape-length distributions are shown in Fig. 6.

Plants grown in short days. 1941-6. Variety 'Cambridge Stock 33'

These were originally set up as a demonstration of plants which had never been allowed to bulb and no records were kept of leaf production. Seed of Cambridge Stock 33 was sown on 23.9.41 in natural day-lengths in the cool

¹ This plant was not included in the first sampling (Table V).

greenhouse. Later 8 of the 24 plants produced were pricked out singly into thumb-pots, the rest into two large flower-pots, 8 plants in each, and later into a large box. After the spring equinox of 1942 the day-length received by these plants was kept at 11 hours during the summer by covering with a light-proof wooden box. By the end of the year the plants in thumb-pots were already much smaller than those grown in the box. During the winter of 1942-3 the plants were again exposed to natural day-lengths and the light-proof box was again made use of after the spring equinox of 1943. The first inflorescence appeared in the largest plant of all on 3.4.43 and three more of the larger plants had bolted by 12.5.43. On 13.5.43 a second inflorescence had been produced by the first plant to flower. Five other plants bolted by 4.6.43 and bolting then ceased. Assuming an average rate of emergence of one leaf per fortnight, an estimate based on the records kept in many other experiments, the first inflorescences would have been produced after 35 or 40 leaves. A few of the smaller plants were still vegetative, and of the plants which had bolted a few died after flowering as no axillary bud appeared to continue the production of fresh leaves. The plants remaining at the autumn equinox were transplanted separately into tins such as were used in the experiments described above and were exposed to natural day-lengths again in the winter. During 1944 all the seventeen remaining plants bolted, the first inflorescence appearing on 10.4.44 and the last on 30.5.44. Two plants produced a second inflorescence each during this period. After flowering only a few plants produced new vegetative growth and the rest therefore died. A few more plants were lost by fungal attack during the winter and only three remained to flower again in 1945—two on 4.4.45 and the remaining one next day. Only one plant survived into 1946, and after bolting on 18.4.46 this, too, failed to produce more leaves and later died.

At no time were any bulbs produced and the continued growth produced leek-like plants of remarkable size with tall, stout pseudostems.

GENERAL DISCUSSION

i. Inflorescence initiation

There is no evidence in any of these experiments that inflorescence initiation was directly affected by day-length. On the other hand, the dominant factor controlling initiation would seem to be temperature (Table VI).

In general it would appear that none of the varieties used in these experiments could initiate inflorescences when growing or lying dormant in temperatures greater than about 17° C. Thus, in Expts. I, IV, and VI, where plants were grown in two temperatures, no inflorescences emerged and no signs of suppressed inflorescences could be found on dissection in any of the plants grown in the warm greenhouse, although this had not a controlled temperature and during the winter months the average temperature fell to about 20° C. Again, with a single exception (in Expt. IV) no plant in the cool greenhouse produced inflorescences in the height of the summer. The plants

of Stock 33 which were kept in continuous short days did bolt as late as 4 June (in 1943) but the last of these inflorescences would have been *initiated* in March about 10 weeks previously (assuming five leaves unemerged at the time of its formation as were found in dissections of the short-day plants in the other experiments). In this particular experiment the plants bolted during a short season at about the same time each year and some plants produced more than one inflorescence during that season. This indicates strongly that some annual variate, such as temperature, was controlling inflorescence initiation (or emergence).

TABLE VI

Average temperature in Expts. I, IV, V, and VI, divided into summer and winter portions (° C.)

Temperatures for periods in which inflorescence initiation took place are shown in **heavy type**

						High temperature	Low temperature
<i>Experiment I (sets)</i>							
Summer,	17.3.42–6.10.42	.	.	.	.	26.2	18.2
Winter,	7.10.42–26.1.43	.	.	.	.	24.4	13.8
<i>Experiment IV (seed)</i>							
Winter,	9.8.43–10.3.44	.	.	.	.	—	13.8
Summer,	{ 3.3.44–8.9.44	.	.	.	.	25.4	—
	{ 11.3.44–8.9.44	.	.	.	.	—	17.9
Winter,	9.9.44–23.2.45	.	.	.	.	21.9	12.4
<i>Experiment V (sets)</i>							
Summer,	13.4.44–9.8.44	.	.	.	.	—	18.6
Winter,	10.8.44–9.2.45	.	.	.	.	—	13.3
<i>Experiment VI (seed)</i>							
Winter,	{ 13.9.44–21.3.45	.	.	.	.	22.2	11.6
	{ 13.9.44–21.3.45	.	.	.	.	—	12.7
	(Treatment 2)						
Summer,	22.3.45–3.10.45	.	.	.	.	24.6	18.7
Winter,	4.10.45–12.2.46	.	.	.	.	19.9	12.0

The largest number of unemerged leaves found on dissection to precede an inflorescence initial was 5 in any of these experiments. About one new leaf emerged every 2 weeks in the winter, e.g. an average of 5.6 leaves emerged in the 10 weeks following transfer to long days in Expt. VI and 4.6 leaves over the same period in short days; thus the date at which the first inflorescence initial was laid down would be about the middle of November in Expt. VI and the middle of October in Expt. IV. Indeed, from all the experiments it would seem that initiation began almost as soon as the temperature began to fall in the autumn (Fig. 1). On the other hand, newly initiated inflorescences have been found in most experiments as late as January or February. The reason for initiation being spread over such a long period is considered below in connexion with the size effect.

It is to be expected that varieties will differ in respect of the maximum temperature under which initiation can occur, but from these and other

experiments carried out in the Research Institute it appears that varieties used in Britain are tolerant of temperatures of about the same level. Varieties used in the tropics seem able, however, to initiate inflorescences at much higher temperatures. Thus, in a personal communication the late F. Crowther informed the authors that in the Sudan native onion varieties bolted freely while English varieties growing alongside remained vegetative indefinitely.

The importance of the size effect in contributing to the stage of 'ripeness to flower' (Gregory, 1936) in the onion plant has been noticed previously in experiments upon the bolting behaviour of onions grown from sets (Thompson and Smith, 1938; Heath, 1943*a*; Blaauw *et al.*, 1944; Holdsworth, 1945*a*; Heath *et al.*, 1947). As it is intended to make this the subject of a separate communication the phenomenon will only briefly be commented on here and only in relation to plant (rather than bulb) size. Treatment 6 of Expt. IV was intended specifically to test the effect of time of sowing on bolting, and as, in fact, the plants of this treatment arrived at the flowering stage only shortly after the comparable plants of treatment 1, which had been sown seven months earlier, it was to be expected that a conclusion as to the importance of plant size might be reached. However, the actual size of the plants in the two treatments did not differ greatly at the time of pickling nor was the number of inflorescence initials very different. The younger plants did, however, form inflorescences at a considerably lower leaf number—the average number of leaves preceding the inflorescence was 24.2 ± 1.1 in treatment 6 and 34.3 ± 2.8 in treatment 1. Thus it seems that, at any rate in treatment 1, the minimum leaf number and size to flower had been greatly exceeded. The simplest explanation of the fact that in all experiments except treatment 6 of Expt. IV bolting began in the *second* winter only appears to be that the minimum size necessary was only attained in the preceding summer at a time when the temperature was unfavourable. Likewise the results of Expt. IV indicate that size *as such* (possibly as a resultant of integrated leaf area) rather than leaf number or age is the determining factor as to whether any plant shall remain vegetative or bolt. The application of the same hypothesis conveniently explains the behaviour of the plants of Stock 33 kept in short days throughout. The first plant to bolt did so after two winters. This was the largest plant in the experiment and in that season the smaller plants did not all bolt. With the onset of high-temperature conditions in the summer bolting ceased until the same time in the following year. All the plants then bolted, having by then passed the minimum size necessary. Again, with the coming of summer initiation ceased until the following spring. It is very probable that in each experiment size was a determining factor in deciding at what time any particular plant was going to bolt after the temperature fell in the autumn.

In every experiment it was apparent that inflorescences first appeared in the largest plants whatever their leaf number and there is no evidence that the plants which failed to initiate inflorescences did so because a minimum leaf (node) number had not been reached. In fact in Expt. VI ('Brown Globe' Table VII) the average leaf numbers of the plants which did not produce

inflorescences exceeded the average number of leaves preceding the inflorescences in those that did.

TABLE VII

Experiments IV and VI. Total number of leaves produced in plants which did and did not flower by end of experiment

	With inflorescences		Without inflorescences	
	Stock 28	Brown Globe	Stock 28	Brown Globe
Short days	34.3	28.8	29.7	32.3
Long days in winter	34.1	29.4	36.0	35.5

The leaf number at which inflorescence initiation can take place in stored bulbs (Heath and Mathur, 1944) is very much lower than those found here; in the case of onion sets as low as 13 or 14 including scales. In a strain of 'Ailsa Craig', Mathur (unpublished) found that plants grown from seed in the open could bolt in the early summer a few months from germination, and here again the leaf number before the inflorescence was about 13. The variety in question was known to be a bolting strain and also the conditions of growth had been made very favourable so that the plants were very large at this leaf number. Thus 13 may be the lowest leaf number at which initiation can take place whatever the size of the plant and whatever the temperature. Under the conditions of the experiments now under review the size effect and the higher average temperature even in the cool part of the greenhouse presumably prevented initiation at such an early stage. The single plant in Expt. IV which bolted in July had already produced 23 leaves and presumably was not large enough to bolt at the time it had attained the minimum leaf number (April 1944).

There is no evidence in the data from any of the experiments that bulb formation directly affects inflorescence initiation. Under normal conditions inflorescence initiation takes place in early spring in the dormant bulb, but in addition some further initiation often occurs after planting. In the experiments described here initiation occurred both in dormant bulbs among the continuous long-day plants of Expts. IV and VI and also in growing plants, both those which had never bulbed at all (continuous short-day plants) and those which had bulbed at some period previously.

In Expts. II and IV the percentages of plants with inflorescences were apparently no greater if the plants had been allowed to bulb previously, either if kept in long days throughout the experiment or if returned to short days after bulbing. In Expt. II, in fact, a few of the bulbed plants did not produce inflorescence initials whereas all the short-day plants did. It is interesting, however, to note that previous bulbing reduced the number of emerged leaves preceding the inflorescence. This would presumably be a direct effect of the cessation of leaf production during bulbing and is evidence that total emerged leaf number was not, in these experiments, a factor determining the onset of initiation.

In Expt. VI the percentage of plants with inflorescence initials on 9.4.46 was found to be much less in the 'long days during final winter' (3) than in the short-day treatment (1). In previous experiments (I, IV, V) it had been found that the percentage in these treatments was not materially different and this has been cited as evidence that initiation was not affected by day-length. The data for the earlier sampling of this experiment (12.2.46) support the same view, the respective percentages then being 59 and 68 (Table 5). In fact, in the long-day treatment the percentage of plants with inflorescences had, two months later, apparently *declined*. It was noted by Heath and Mathur (1944) that where the percentage of sets with inflorescence initials at planting time was known, the whole of these inflorescences could not be accounted for in the total emerged and suppressed found in the mature bulbs at harvest. An exactly similar result in later experiments (Holdsworth 1945*b*) has led to the view that many of the smaller initials were suppressed so early that it was technically impossible to find them when crushed by the subsequent growth and bulbing of the axillary bud. It seems very probable that this was true of the long-day plants in Expt. VI, and the apparent decrease in initiation in treatment 3 ('long days during final winter') from the first to the second sampling, as well as the lower percentage of inflorescences found in continuous long days (treatment 2) than in continuous short days (treatment 1), would thus be explained. A similar explanation can be applied to the small amount of further initiation found on dissection in Expt. III, where again a large number of plants were bulbed before pickling.

Under appropriate temperature conditions, once the terminal inflorescence has been produced, further inflorescences can arise from axillary buds apparently indefinitely, but each bud produces not less than three leaves before the new inflorescence. Thus the stage of ripeness-to-flower having been reached, the plant can remain permanently in the reproductive condition.

Summarizing these conclusions as to the effects of the factors tested on inflorescence initiation:

- (a) There may be a minimum leaf number below which initiation cannot take place. If so, it is relatively low and was not operative in these experiments.
- (b) There is a minimum plant size below which initiation cannot take place.
- (c) There is a maximum temperature above which initiation cannot take place.
- (d) Day-length does not directly affect inflorescence initiation.
- (e) Bulb formation does not directly affect inflorescence initiation but may destroy inflorescences newly initiated.

ii. *Inflorescence emergence*

The data of bolting (that is, the emergence of inflorescences) represent a composite result of the effects of the factors tested on both inflorescence initiation and subsequent elongation of the scape. Thus, in Expt. I it was found by dissection (Table II) that the percentages of plants which had

initiated inflorescences were exactly equal in the long and short days at low temperature but that no plants at all had initiated inflorescences at high temperature in either day-length. The percentage bolting, however, was 21 per cent. in long days at low temperature and nil, by the end of the experiment, in each of the other three treatments. The complete lack of bolting at high temperature was, therefore, a temperature effect on initiation; at low temperature in the short photoperiod it was a day-length effect on the rate of scape elongation. Similar effects of long days at low temperature in accelerating scape elongation have been found in all the experiments described here. This is most effectively demonstrated by histograms showing the frequency of scapes in various length-classes, as in Figs. 2-6. Allowing for errors in sampling, the distributions for short days throughout are such as might be expected with random variation in the time of onset of initiation. The effect of long days in the final winter tends to widen the distribution so that most inflorescences are much more advanced than the longest in short days, but at the same time a few inflorescences recently initiated are also found. This indicates that the effect of day-length in the winter is only to speed emergence (scape elongation) and that the time of initiation is unaffected. In the short account of Expt. I already published (Heath and Holdsworth, 1943) an alternative hypothesis, viz. that initiation had occurred earlier in long days but that only 65 per cent. of the plants were capable of initiating inflorescences that season, was presented also. The results of the subsequent experiments (Expts. IV, V, VI) make this second hypothesis extremely unlikely as the percentages of inflorescences found have varied from 59 to 97 per cent. and in no case has the difference between the number in long and short days been statistically greater than would be expected by chance variation. In Expt. VI (Table V), for example, where the plants were pickled at two times expressly to test these hypotheses, at the *earlier* sampling the difference between the day-length means was not only small but in the wrong direction and from the later sampling the number of initials in long days appeared to be substantially less than in short days.

The crucial experiment of measuring the elongation of scapes in long and short days in plants in which initials were known to be present was attempted in the spring of 1947. Usually inflorescences are initiated in stored sets too late in the spring for the effect of long days at really low temperatures to be observed in the greenhouse. However, by choosing a bolting variety ('Brown Globe') and examining large bulbs it was possible to choose 40 as early as 6 February in which inflorescence initials were reasonably certain to be present; of these, 30 were planted in short days and 10 in long days. The latter and 10 of the former were pickled on 4 April and the scape lengths of the recovered inflorescences compared before emergence. Of the remaining plants half were transferred to long days when the first inflorescence emerged and subsequent growth of emerged scapes was measured daily (from 30 April). The results of the dissections tend to confirm the hypothesis that elongation is more rapid in long days, but the difference just failed to reach statistical

significance. In short days the average inflorescence length was 1.3 mm.; in long days 2.3 mm. The growth of the emerged inflorescences was not apparently affected by day-length, but the temperature in the greenhouse had risen considerably by the time this part of the experiment could be carried out.

As long days promote bulb formation in the onion plant it is evident that if bulb formation affects scape elongation a further effect of day-length on bolting must be added to the above. All the experiments were terminated during the winter or early spring when the temperature in the cool section of the greenhouse was very low and therefore in plants transferred to long days in the previous autumn bulbing was greatly delayed. On the early bolting, therefore, effects of bulbing can be dismissed. Expt. VI is of special interest for this question as few plants had bulbed by the time of the first sampling, but 91 per cent. of the long-day, low-temperature plants (treatment 3) were bulbed by the end of the experiment. It is known from experiments on the suppression of inflorescences that the pressure exerted by the scales as they swell can crush small inflorescences (that is, not exceeding the length of the bulb itself) and suppress them altogether (see pl. 3, fig. 11, Heath and Holdsworth, 1948). On the other hand, part of the bulbing process is the suppression of leaf elongation in the formation of bulb scales and it is probable that the elongation of the scape is affected in the same way as leaf initials in their conversion into scales. In very few instances have emerged inflorescences been produced by plants after they have been recorded as visibly bulbed, but in many cases swollen leaf bases and *scales* have been found surrounding the emerged inflorescence. Since, in the absence of bulbing, all the leaves normally emerge before the inflorescence it is therefore probable that the degree of bulbing required to prevent leaf elongation is less than that necessary to prevent elongation of the scape.¹ In a few cases swollen scales had obviously been split by the passage of the scape after the scale had ceased growing.

These effects of bulbing on emergence of inflorescences apply where bulbing is taking place at the same time as the inflorescences are developing, but in Expts. II and IV there was an after-effect of long days received in the summer on the emergence of the inflorescences in mid-winter. Plants were allowed to bulb in long days in the summer and were then transferred back to short days in the autumn. Although showing no signs of bulbing in the winter, they yet showed a pronounced delay in the emergence of inflorescences. In Expt. II, by the time the plants were pickled (April) emerged inflorescences had been produced in both treatments, but they were produced earlier in the plants which had been kept always in short days. In Expt. IV, on the other hand, in neither of these two treatments was there any bolting when the experiment was terminated. In both experiments the total number of inflorescences including unemerged initials was similar whether the plants had bulbed or not, but, as shown in Figs. 3 and 4, the average scape length was less if the

¹ Bulbing begins in the emerged leaves and the new leaves are progressively affected from the outside to the centre of the bulb.

plants had previously bulbed. This indicates that either long days themselves, or bulb formation caused by long days, decrease the rate of scape elongation of inflorescences emerging perhaps six months later. The distributions of scape lengths in Fig. 4 for plants which were both allowed to bulb in the summer and exposed to long days in the winter (treatments 2 and 5) rather appear to be bimodal, suggesting perhaps that these two effects of long days are in antagonism but that the effect of long days at low temperatures is most apparent in the later stages of emergence.

The effect of temperature on inflorescence emergence cannot be assessed from these experiments as initiation was completely suppressed in the high temperature treatments (p. 366). Heath and Mather (1944) record, however, that the emergence of inflorescence initials already present in sets was suppressed by growth at temperatures of about 25° C. It would be possible to explain the annual flowering of the Stock 33 plants in continuous short days (p. 366) as an effect of high temperature on scape emergence alone, for there is no definite evidence from the experiment to show whether inflorescence initials were present or not during the period each year when the plants appeared vegetative.

As the relationships between bolting and environmental conditions have in these investigations proved to be so complex, it would be difficult to predict precisely the bolting behaviour of onions grown under natural conditions. However, the combined effect of summer conditions (long days, high temperatures) may be expected to suppress bolting, whereas in spring the conditions are often very favourable to both initiation of inflorescences and emergence. The lengthening days may be expected to accelerate scape elongation as long as the temperatures are low enough to prevent rapid bulbing. Thus, heavy bolting of onions grown from sets or autumn-sown seed may be expected following a cold spring and relatively little bolting after a warm spring and early summer.

TABLE VIII

Effects of day-length, temperature, and bulbing on flowering of the onion plant (general summary)

	Short days		Long days
High temperature	No bulbing	Bulbing	
	No initiation of inflorescences.	No initiation of inflorescences.	
	No emergence of initials formed previously in low temperature.	Initials formed previously are destroyed and do not emerge.	
		Emergence of inflorescences initiated subsequently in low temperature is delayed.	
Low temperature	No bulbing.	Bulbing.	No bulbing.
	Initiation of inflorescences.	Initials formed previously are destroyed.	Initiation of inflorescences.
	Slow emergence.	Initials formed subsequently can emerge on sprouting at low temperature.	Rapid emergence.

The effects of day-length, temperature, and bulbing on inflorescence initiation and emergence discussed in this paper are summarized in Table VIII. An attempt to correlate these and other observations on the flowering and bulbing of the onion plant by means of a speculative hormone hypothesis has recently been published (Heath and Holdsworth, 1948).

SUMMARY

At temperatures above about 17° C. inflorescence initiation in growing onion plants, as in stored sets, is suppressed whether the plants are kept in long or short days. Independently of current day-length and of previous day-length treatment, if the plants are sufficiently large initiation begins very shortly after the temperature falls below *c.* 15° C. Emerged inflorescences appear some ten or so weeks later. Small plants are unable to initiate inflorescences under any of the conditions tested, and actual size (perhaps leaf area) rather than leaf or node number seems to be the important factor. Inflorescence emergence is suppressed at high temperatures in short days or long days; in long days bulb formation also suppresses emergence at lower temperatures. In long days at temperatures sufficiently low for bulbing to be delayed, however, emergence is accelerated. Plants which have produced bulbs in long days in the summer show a delay of inflorescence emergence in the following winter.

LITERATURE CITED

- BLAAUW, A. H., HARTSEMA, A. M., and LUYTEN, I. (1944). Bloemen of Bollen bij *Allium cepa* L. (with summary). III en IV. *Ned. Akad. Wet. Afd. Natuurkunde*, **53**, 274-91.
- GARNER, W. W., and ALLARD, H. A. (1923). Further studies in photoperiodism, the response of the plant to relative length of day and night. *J. Agric. Res.* **23**, 871-920.
- GREGORY, F. G. (1936). The effect of length of day on the flowering of plants. *Sci. Hort.* **4**, 143-54.
- HARTSEMA, A. M. (1947). De periodieke Ontwikkeling van *Allium cepa* L. var. Zittauer Riesen. *Med. Landbouwhoogeschool, Wageningen*, **48**, 265.
- HEATH, O. V. S. (1943*a*). Studies in the physiology of the onion plant. I. An investigation of factors concerned in the flowering ('bolting') of onions grown from sets and its prevention. Part 1. Production and storage of onion sets and field results. *Ann. Appl. Biol.* **30**, 208-20.
- (1943*b*). Studies in the physiology of the onion plant. I. An investigation of factors concerned in the flowering ('bolting') of onions grown from sets and its prevention. Part 2. Effects of day length and temperature on onions grown from sets, and general discussion. *Ibid.* **30**, 18-19.
- (1945). Formative effects of environmental factors as exemplified in the development of the onion plant. *Nature*, Lond., **155**, 623.
- and HOLDSWORTH, M. (1943). Bulb formation and flower production in onion plants grown from sets. *Ibid.* **152**, 334.
- (1948). Morphogenic factors as exemplified by the onion plant. *Soc. Exp. Biol. Symp.* **2**, Growth, 326-50.
- TINCKER, M. A. H., and BROWN, F. C. (1947). Studies in the physiology of the onion plant. III. Further experiments on the effects of storage temperature and other factors on onions grown from sets. *Ann. Appl. Biol.* **34**, 473-502.
- and MATHUR, P. B. (1944). Studies in the physiology of the onion plant. II. Inflorescence initiation and development, and other changes in the internal morphology of onion sets, as influenced by temperature and day length. *Ibid.* **31**, 173-86.

- HOLDSWORTH, M. (1945*a*). A comparative study of onion varieties in relation to bolting and yield when grown from sets. *Ibid.* **32**, 22-34.
- (1945*b*). *An investigation of the physiological processes affecting bulbing and flowering in the onion*. Ph.D. Thesis, Univ. of London.
- MAGRUDER, R., and ALLARD, H. A. (1937). Bulb formation in some American and European varieties of onions as affected by length of day. *J. Agric. Res.* **54**, 719-52.
- MCCLELLAND, T. B. (1928). Studies in the photoperiodism of some economic plants. *Ibid.* **37**, 603-28.
- PURVIS, O. N., and GREGORY, F. G. (1937). Studies in the vernalisation of cereals. I. A comparative study of vernalisation in winter rye by low temperature and by short days. *Ann. Bot., N.S.*, **1**, 569-91.
- SCULLY, N., PARKER, M. W., and BORTHWICK, H. A. (1945). Interaction of nitrogen nutrition and photoperiod as expressed in bulbing and flower stalk development of the onion. *Bot. Gaz.* **107**, 52.
- THOMPSON, H. C., and SMITH, O. (1938). Seedstalk and bulb development in the onion (*Allium cepa* L.). *Bull. Cornell. Agric. Exp. Sta.* No. 708, 21 pp.

